EXHIBIT A

Proc. Natl. Acad. Sci. USA
Vol. 76, No. 11, pp. 5616–5620, November 1979
Biochemistry

Structural studies of "active complex" of bleomycin: Assignment of ligands to the ferrous ion in a ferrous–bleomycin–carbon monoxide complex

(¹H NMR/mechanism of action/DNA binding/DNA degradation/antitumor agent)

NORMAN J. OPPENHEIMER*, LUIS O. RODRIGUEZ†‡, AND SIDNEY M. HECHT†‡

*Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143; and †Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Communicated by John Ross, August 2, 1979

ABSTRACT **Proton NMR studies at 360 MHz establish the binary Fe(II)-bleomycin complex to be paramagnetic with a spectrum covering 70 ppm. Addition of carbon monoxide generates a stable, diamagnetic Fe(II)-bleomycin-CO complex that is a putative structural analog of the "active" Fe(II)-bleomycin-O_2 complex. The following six groups have been determined to be coordinated to the Fe(II) ion from analysis of the highly resolved ¹H NMR spectra of this complex: CO, the primary and secondary amine nitrogens of the β-aminoalanine moiety, the carbamoyl moiety on the 3-position of mannose, the pyrimidine N-1, and the imidazole N-1. The Fe(II)-bleomycin-CO complex binds to DNA, as shown by fluorescence quenching experiments, but Fe(II)-bleomycin-CO does not mediate thymine release. These results necessitate a major revision in the current model for metal coordination to bleomycin.**

The bleomycins (Fig. 1) are a class of glycopeptide antibiotics with strong antineoplastic activity (1). They are isolated from *Streptomyces verticillus* as copper(II) complexes (2) and also show high affinities for the divalent ions of iron, nickel, cobalt, and zinc. The Fe(II)–bleomycin complex, in conjunction with reducing agents and oxygen, causes the degradation of DNA via strand scissions with the concomitant release of free bases (3–5). A two-step mechanism has been suggested for this reaction, involving the intercalation of the bithiazole moiety into DNA followed by the decomposition of a labile Fe(II)–bleomycin–O_2 complex to generate Fe(III)–bleomycin and either hydroxyl or superoxide radicals that can then degrade the DNA (6, 7). Investigations into the structural properties of the Fe(II)–bleomycin–O_2 complex are hampered by its rapid decomposition (8, 9). NMR studies have focused primarily on the binary complex with diamagnetic Zn because it yields highly resolved spectra (10–13). Obviously the coordination in binary complexes must differ from the "active" Fe(II)–bleomycin–O_2 complex because O_2 has become a ligand. According to the current model, this is accomplished by O_2 coordinating to Fe(II) in place of the 3-carbamoyl moiety on the mannose (7). This model, however, has serious potential deficiencies. It is based on an extrapolation from the x-ray structure of a *binary Cu(II) complex* of a putative biosynthetic *precursor* of bleomycin designated P-3A (14), a compound that lacks many of the ligating atoms ostensibly involved in the binding of metals to bleomycin. The potential ramifications of these differences in ligation on the physiochemical properties of bleomycin and its interaction with DNA make the development and study of a suitable structural analog of the Fe(II)–bleomycin–O_2 complex imperative. Based on the perceived analogy with the properties of Fe(II)–porphyrins such as in hemoglobin or cytochrome *P*-450 towards O_2, we have examined the properties of Fe(II)–bleomycin in the presence of carbon monoxide, a compound that forms stable structural analogs of Fe(II)–porphyrin–O_2 complexes. In this paper we report: (*i*) that Fe(II)–bleomycin readily forms a stable, diamagnetic complex with CO; (*ii*) an analysis of the highly resolved ¹H NMR spectra of the Fe(II)–bleomycin–CO complex; (*iii*) the assignment of the ligands to the metal based on the ¹H NMR data; (*iv*) that the Fe(II)–bleomycin–CO complex readily binds to DNA, as studied by fluorescence quenching; and (*v*) that Fe(II)–bleomycin–CO does not mediate the release of thymine from DNA.

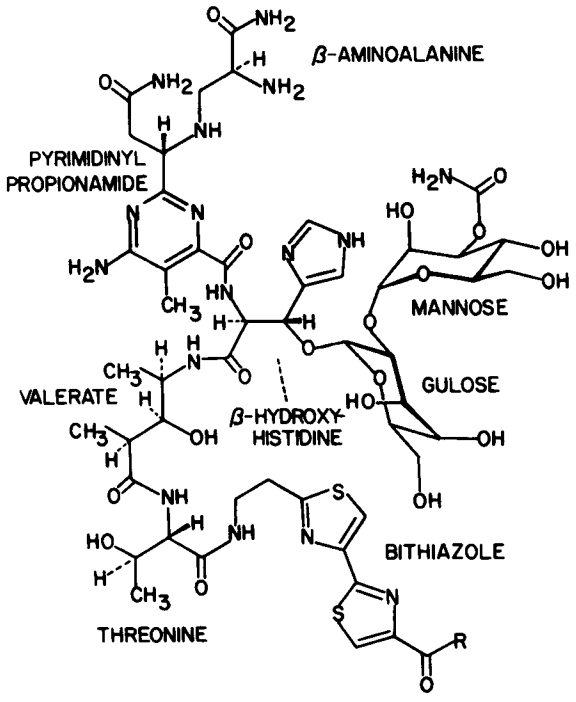


FIG. 1. Revised structure of bleomycin. R = $NH(CH_2)_3S^+(CH_3)_2$, bleomycin-A2; R = $NH(CH_2)_4NHC(NH)NH_2$, bleomycin-B2.

MATERIALS AND METHODS

The clinical mixture of bleomycin-A2 and -B2, blenoxane, was obtained from the National Cancer Institute. Bleomycin-A2 was isolated from this mixture by elution through a Sephadex C-25 column with an ammonium formate gradient (0.05–0.5 M), pH 6.4, as described (15). The appropriate fractions were concentrated to dryness under diminished pressure, and the excess ammonium formate was removed by sublimation (0.03

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

‡ Present address: Department of Chemistry, University of Virginia, Charlottesville, VA 22901.

mm Hg at 30°C for 8–10 hr) followed by elution through a Sephadex G-10 column with 0.02 M ammonium bicarbonate. Final desalting was then accomplished by repeated evaporations of its aqueous solution under diminished pressure. The ^1H NMR and fluorescence spectra of this material and its ability to effect release of thymine from DNA were unchanged as a result of the separation.

^1H NMR Spectra. *NMR parameters.* Spectra were obtained at 360 MHz on a Bruker HXS-360 NMR spectrometer equipped with a Nicolet Technologies 1180 computer/Fourier transform system and a computer-controlled homonuclear decoupling accessory. Quadrature detection was used and both 16K and 32K Fourier transforms were obtained with a spectral width of 3610 Hz. The Fe(II)–bleomycin spectrum was obtained with a spectral width of 36,000 Hz and a 32K Fourier transform was used. Typically, 128 transients were accumulated with 2.7 sec between pulses. Equilibrium intensities were observed for all the resonances under these conditions except those of the bithiazole moiety.

Sample preparation. Bleomycin (2 μmol determined spectrophotometrically) was twice lyophilized from 99.8% ^2H_2O and then dissolved under N_2 in 0.37 ml of 100% ^2H_2O (paramagnetic-free, Aldrich) in a septum-sealed NMR tube. $FeSO_4$ (2 μmol as 10 μl of a 0.2 M solution in 100% ^2H_2O) was added anaerobically (by syringe) followed by 1.95 μmol of NaO^2H, yielding a pale pink solution with a final concentration of 5 mM. Exposure to air (oxygen) caused a rapid color change to tan, which could be completely reversed by addition of dithionite. The CO adduct was formed by freezing the Fe(II)–bleomycin solution, evacuating the NMR tube, and then allowing the tube to fill with CO. Upon thawing, the solution became bright yellow. The complex was quite stable and could be lyophilized without apparent loss of bound CO. Exposure to air, however, led to the same rapid formation of a tan solution as was observed with Fe(II)–bleomycin. The ^1H NMR spectrum of neither Fe(II)–bleomycin nor Fe(II)–bleomycin–CO was affected by the presence of dithionite even at 5-fold molar excess. Therefore, 0.2 μmol of dithionite was added routinely to the samples to protect against traces of O_2. The p^2H of the solutions was measured in the NMR tube subsequent to spectral acquisition with an Ingold 4-mm-diameter glass electrode. A stream of nitrogen was directed at the NMR tube to prevent any oxidation of the sample until after the measurement had been made. The standard electrode correction has been used, p^2H = meter reading + 0.4. The internal standard, 3-trimethylsilyl sodium [2,2,3,3-$^2H_4$]propionate, was used.

Fluorescence Quenching of Bleomycin-A2 by DNA. Calf thymus DNA (from Calbiochem) was dissolved in 0.1 M NaCl and dialyzed exhaustively at 4°C, first against a 0.1 M NaCl solution containing 0.1 M EDTA and then against 0.1 M NaCl. The concentration of the DNA was determined from its absorbance at 260 nm. All measurements were conducted with deionized water freshly distilled from EDTA.

Fluorescence measurements were performed on a Farrand Mark I Spectrofluorimeter with emission and excitation correction. The excitation wavelength was 295 nm; a 10-nm slit width was used throughout. Measurements were made at 25°C in a septum-stoppered cell. The atmosphere was changed by bubbling the desired gas through each solution for at least 1–2 min. Solutions were made <24 hr in advance of the experiments and stored at <−20°C; the pH of each solution was adjusted to 5, 7, or 9 with aqueous NaOAc or Tris·HCl to a final concentration of 5 mM. Glutathione was added to a final concentration of 0.034 mM to prevent formation of Fe(III); its addition had no effect on the fluorescence spectra. Bleomycin-A2, Fe(II), and DNA were added to final concentrations of 0.017–0.020, 0.034, and 0.71 mM, respectively. The $Fe(NH_4)_2(SO_4)_2$ solution (previously degassed) was added to the sample via syringe under continuous N_2 bubbling.

Spectra of Fe(II)–bleomycin-A2 under N_2 with and without DNA were run on samples prepared by degassing the Fe(II)-free samples, adding the deaerated Fe(II) solution, and further degassing the combined solution. To show the effect of CO, we ran the spectra on the same samples after introduction of CO. These samples were then opened to the air, allowed to equilibrate for about 5 min, and used to record the spectra of the oxygenated species. As a control, spectra were also recorded on samples of Fe(II)–bleomycin-A2 (with and without DNA) prepared in solutions that had been flushed with CO rather than with N_2.

Release of [^3H]Thymine from PM-2 DNA by Bleomycin. PM-2 DNA (containing [^3H]thymine, specific activity 21 × 10^6 cpm/mol) was prepared as described (16). Bleomycin activity was measured in terms of the release of [^3H]thymine from acid-insoluble products. The reaction mixture (25°C, 0.60 ml total volume) consisted of a solution of 0.05 M sodium cacodylate (pH 6.9) containing 68 μM glutathione, 1.15 μM PM-2 DNA (15,000 cpm of [^3H]thymine), 34 μM $Fe(NH_4)_2(SO_4)_2$, and 34 μM bleomycin in a septum-stoppered vial. Fe(II), followed by bleomycin, were added last (from CO-flushed stock solutions) after the reaction mixture was purged with deoxygenated CO for 10 min. At predetermined time intervals, 50-μl aliquots were removed and applied to glass fiber disks that had been soaked with thymine and 10% aqueous trichloroacetic acid solutions. After 3.5 min of incubation, the reaction mixture was opened to the atmosphere and additional aliquots were taken and applied to glass fiber disks. The dried disks were washed thoroughly with 5% aqueous trichloroacetic acid, dried, and used for determination of radioactivity.

RESULTS

^1H NMR studies

The binary complex of Fe(II)–bleomycin yielded the remarkable ^1H NMR spectrum shown in Fig. 2. The contact and pseudocontact shifts arising from the paramagnetic iron atom spread the resonances over a 70-ppm range. The magnitude and direction of the shift induced for a given resonance by a paramagnetic center have a defined angular and distance dependence. Therefore, a complete assignment of the resonances will provide, in principle, detailed conformational and structural information. Analysis of this spectrum was complicated by the slow rate of exchange we observed between Fe(II)–bleomycin and metal-free bleomycin which precluded the use of saturation-transfer methods (17) for assigning the resonances of the complex. We are, therefore, exploring alternative methods for establishing definitive assignments of the resonances. We note, however, the absence of line broadening or shifts for the sulfonium methyls in bleomycin-A2. Furthermore, the pattern of resonances centered at 8 ppm was similar to that for the bithiazole moiety in metal-free bleomycin. These results are consistent with previous observations (15, 18) that the bithiazole and terminal cationic groups are spatially remote from the metal binding site.

Addition of CO to the Fe(II)–bleomycin complex caused dramatic changes in the spectral properties of the latter. The ^1H NMR spectrum of the Fe(II)–bleomycin–CO complex (Fig. 3) became highly resolved, with linewidths comparable to those for the Zn–bleomycin complex. The narrow lines established that coordination of CO to the paramagnetic Fe(II)–bleomycin complex leads to a low-spin, diamagnetic iron in Fe(II)–bleomycin–CO. The possibility that the CO merely stripped away the metal from bleomycin could be excluded because the observed chemical shifts in no way resembled the pattern for metal-free bleomycin at any p^2H.

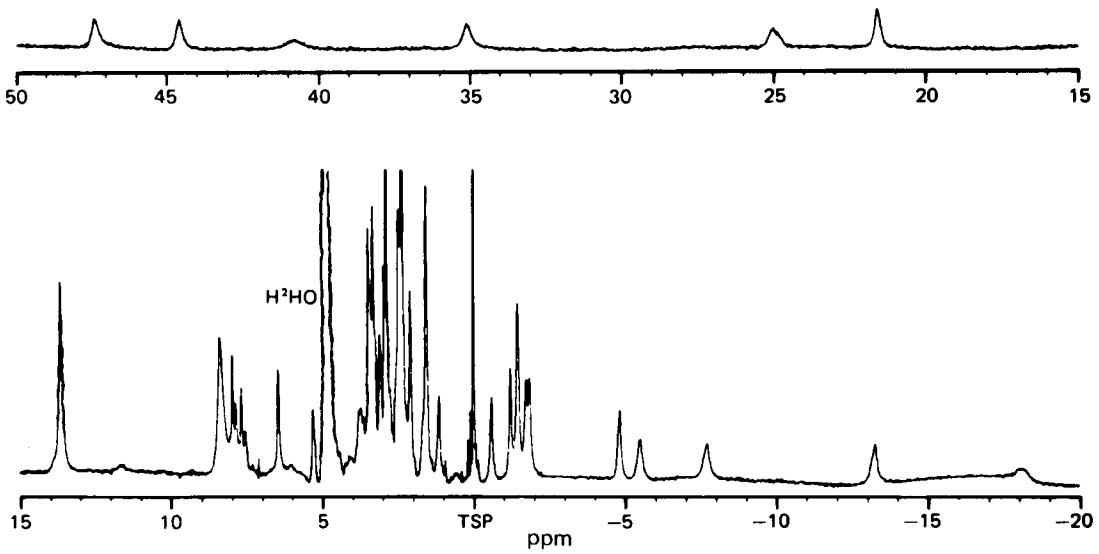


FIG. 2. The 360 MHz ^1H NMR spectrum of the Fe(II)–bleomycin complex obtained in ^2H_2O at 27°C. TSP, trimethylsilyl propionate.

The resonances of Fe(II)–bleomycin–CO have been assigned by analogy to the chemical shifts and coupling constants observed in the Zn–bleomycin complex (13) and by homonuclear spin decoupling experiments. Portions of the ^1H NMR spectra of Zn–bleomycin (p^2H 6.1) and Fe(II)–bleomycin–CO (p^2H 7.0) are compared in Fig. 3; the chemical shifts for selected resonances are listed in Table 1.

Coordination to a diamagnetic metal, like protonation, can result in downfield shifts of the proton resonances adjacent to the site of binding due to withdrawal of electron density. Alternatively, coordination can cause either upfield or downfield shifts due to changes in the juxtaposition of substituents. In either case, the primary change in magnetic environment will be in the immediate vicinity of the metal. Therefore, those resonances with altered chemical shifts should reflect the general region where the metal binds.

As shown in Table 1, formation of Zn–bleomycin caused chemical shift changes relative to metal-free bleomycin that were >0.1 ppm for the following substituents: β-aminoalanine, pyrimidinylpropionamide, pyrimidine methyl, β-hydroxyhistidine, mannose 3′CH, and methylvalerate. In general, formation of Fe(II)–bleomycin–CO affected the same substituents although the magnitudes could differ. The mannose 3′CH, the imidazole, the propionamide, and the pyrimidine methyl groups had chemical shifts similar to those for the corresponding resonances in the spectrum of Zn–bleomycin (13). In contrast, the spectral pattern of the β-aminoalanine group differed considerably from that of the zinc complex. The large nonequivalence of the β-CH_2 resonances observed for Zn–bleomycin was greatly diminished in Fe(II)–bleomycin–CO, and the vicinal coupling between the α-CH and β-CH_2 changed from two coupling constants of 2.0 and 3.1 Hz to 6.8 and 3.7 Hz, respectively. The valerate α-CH resonance was also strongly affected. It was deshielded by 0.40 ppm relative to Zn–bleomycin and had a chemical shift comparable to that found in metal-free bleomycin.

The vicinal coupling constants for the α- and β-CH resonances of the β-hydroxyhistidine moiety in Fe(II)–bleomy-

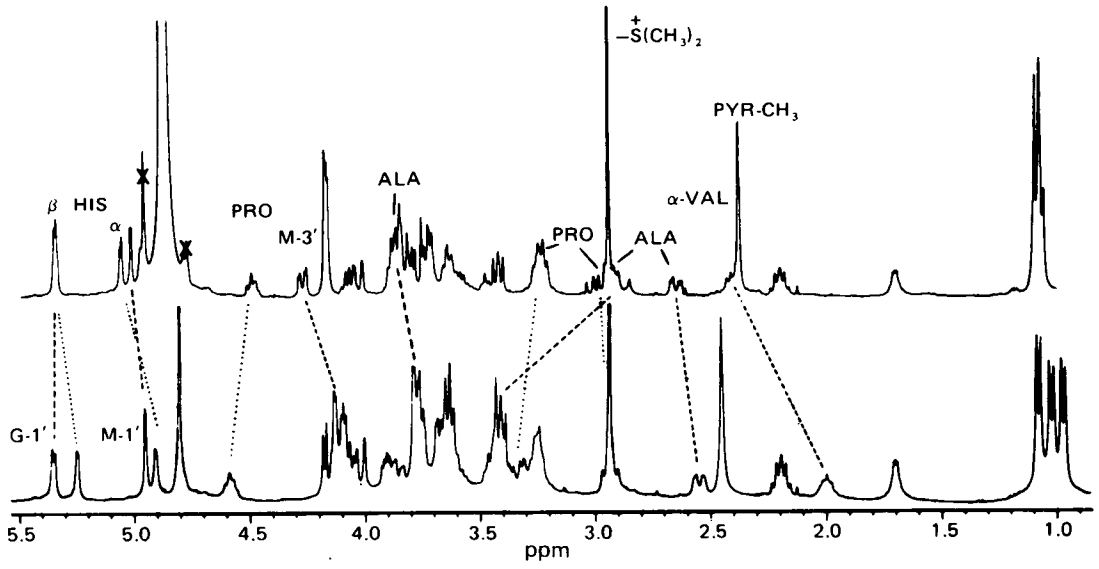


FIG. 3. Comparison of a portion of the 360 MHz ^1H NMR spectra of the Fe(II)–bleomycin–CO complex (*Upper*) with the Zn–bleomycin complex (*Lower*). HIS, hydroxyhistidine; PRO, propionamide; ALA, β-aminoalanine carboxamide; VAL, methylvalerate; PYR, pyrimidine.

Table 1. Chemical shift of selected resonances in bleomycin complexes*

Component group†		Fe(II)–CO	Zn	Metal-free
Bithiazole	A2	8.248	8.251	8.233
		7.996	8.080	8.041
	B2	8.199	8.207	8.190
		7.973	8.059	8.021
Imidazole	2H	7.985	8.095	7.826
	4H	7.372	7.390	7.294
PRO	CH	4.487	4.608	3.988
	CH_2	3.25 ± 0.02	3.364	2.737
		2.953	2.922	2.662
ALA	CH	3.88 ± 0.02	3.79 ± 0.02	3.892
	CH_2	2.929	3.42 ± 0.02	(2.966)
		2.652	2.561	
VAL	αCH	2.416	2.013	2.484
	βCH	3.68 ± 0.02	3.46 ± 0.02	3.731
	γCH	3.71 ± 0.02	3.69 ± 0.02	3.892
PYR-CH_3		2.385	2.469	2.044
MAN 3′CH		4.268	4.09 ± 0.02	4.695

* Chemical shifts are reported in ppm from trimethylsilyl sodium propionate and are accurate to ±0.002 ppm unless otherwise noted. The p^2H of the solutions were as follows: Fe(II)–bleomycin–CO, p^2H 6.4; Zn–bleomycin, p^2H 6.1; bleomycin, p^2H 7.0. All spectra were obtained at 27°C.

† See legend to Fig. 3.

cin–CO were identical to those observed in Zn–bleomycin, thus indicating a <10% contribution from the *trans* rotamer (13). Therefore, the conformation of this portion of the metal–bleomycin complex was little affected by the change of ligands. The conformational changes in the β-aminoalanine moiety, on the other hand, were quite large. In Zn–bleomycin, the *gauche-gauche* orientation predominated (>90%) whereas in Fe(II)–bleomycin–CO either a mixture of *gauche-trans* and *gauche-gauche* orientations was present or there was a fixed, *gauche-trans* geometry that was not fully staggered. Coordination of a metal ion to both amine nitrogens, although precluding free rotation, need not prevent interconversion of conformers.

DNA binding studies

The ability of the Fe(II)–bleomycin–CO complex to bind to DNA was assayed in terms of the fluorescence quenching of the bithiazole moiety (15). As shown in Table 2, the apparent affinity of the Fe(II)–bleomycin–CO complex for DNA was no different from that of Fe(II)–bleomycin at three different pH values. Repetition of the experiment with blenoxane gave the same result (data not shown). Also studied was the degradation of DNA by the Fe(II)–bleomycin–CO complex. As shown in Fig. 4, no thymine release from DNA by this complex was observed. When the complex was exposed to O_2, replacement of CO by O_2 occurred rapidly, as may be seen by the subsequent release of thymine from the PM-2 DNA.

DISCUSSION

Takita *et al.* (7) have proposed a model for the active form of bleomycin based on the x-ray structure of a putative biosynthetic intermediate of bleomycin designated P-3A (14). According to this model, in the absence of oxygen the metal is coordinated to bleomycin via the following six ligands: the primary and secondary amines of the β-aminoalanine, the pyrimidine N1, the N^{α} of the β-hydroxyhistidine, the imidazole N1, and the carbamoyl moiety on the 3-position of mannose (7, 19). In order for this complex to coordinate oxygen, one of the ligands must be released, with the carbamoyl moiety suggested as the exchangeable ligand because the metal binding species, P-3A, lacks both sugar moieties and, therefore, this group.

Table 2. Fluorescence quenching of bleomycin by calf thymus DNA*

Bleomycin-A2, μM	Atmosphere	% fluorescence quenching†
pH 4.7‡		
17	N_2	44
17	CO	42
17	O_2	48
pH 6.8‡		
20	N_2	50
20	CO	47
20	O_2	44
pH 8.4‡		
17	N_2	48
17	CO	46
17	O_2	49

* Carried out in the presence of 0.034 mM Fe^{2+}. Fluorescence emission was recorded at 353 nm.

† In the presence compared to the absence of 0.71 mM calf thymus DNA.

‡ Final pH of the complete incubation mixture.

The ^1H NMR spectrum of Fe(II)–bleomycin–CO, however, clearly shows that the shielding of the mannose 3′CH resonance is comparable to that observed in the binary Zn–bleomycin complex. The sensitivity of the mannose 3′CH resonance to the coordination of Fe(II) and CO is difficult to explain if it is not participating as a ligand because otherwise the sugars should be far removed from the metal binding domain. This is borne out in metal-free bleomycin, where the mannose resonances show no dependence on the state of protonation of either the imidazole or β-aminoalanine moieties (unpublished data). We therefore conclude that the carbamoyl group is a ligand in the Fe(II)–bleomycin–CO complex. The participation of the carbamoyl moiety in the ternary complex explains the importance of this group for DNA strand scission. Isobleomycin (2-carbamoylmannose) shows a considerable decrease in *in vitro* activity relative to bleomycin (20, 21).

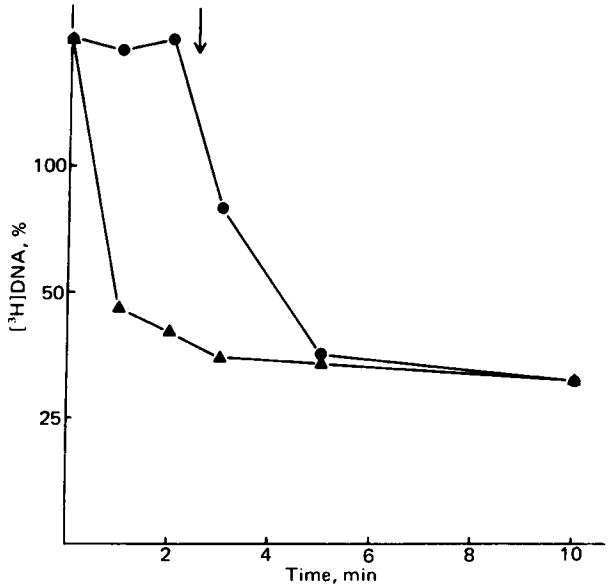


FIG. 4. Release of [^3H]thymine from PM-2 DNA incubated in the presence of blenoxane, Fe(II), and CO (●) or O_2 (▲). After 2.5 min, the sealed incubation mixture containing CO was opened to the atmosphere and additional aliquots were taken for analysis of thymine release.

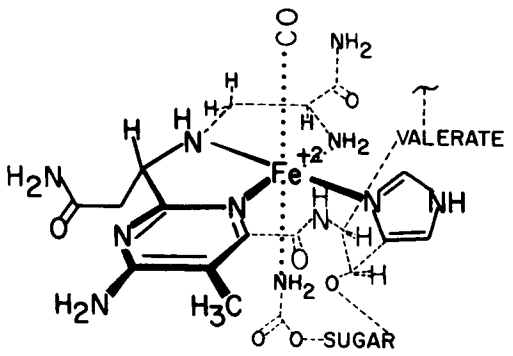


FIG. 5. Schematic drawing of the arrangement of the ligands in Fe(II)–bleomycin–CO. The ligands for which the relative geometry is known are drawn in dark, solid lines. The remaining three ligands (the primary amine, the carbamoyl, and the CO) for which the relative orientations cannot as yet be determined are shown connected by dashed lines. Although octahedral coordination is drawn, the absence of any symmetry should lead to significant deviations of the six ligands from a perfect octahedral geometry.

The remaining ligands to iron in Fe(II)–bleomycin–CO can be assigned from their chemical shift properties relative to bleomycin. The two amines and the imidazole are coordinated to the iron; their chemical shifts are independent of p^2H over the range from 4.5 to 10, and the pyrimidine methyl shows a strong downfield shift comparable to that reported for Zn–bleomycin (10–13). Therefore, these four groups, together with the 3-carbamoyl group and the CO, account for all six ligands in Fe(II)–bleomycin–CO *without invoking coordination to the N^{α} of the β-hydroxyhistidine group.*

A tentative arrangement of the groups coordinated to the Fe(II) ion in Fe(II)–bleomycin–CO is shown in Fig. 5. Model building and preliminary results using distance geometry calculations (22) indicate that a *trans* orientation between the secondary amine and the imidazole is favored; coordination of the pyrimidine and imidazole ligand *trans* to each other is precluded in the absence of coordination to N^{α} of the β-hydroxyhistidine (G. M. Crippen and N. J. Oppenheimer, unpublished data). The pyrimidine moiety, as the third ligand, will then be *cis* to both the secondary amine and the imidazole, as shown in Fig. 5. The arrangement of the remaining three ligands, however, cannot be determined unequivocally. This arrangement of ligands would exclude an alternative arrangement, analogous to that found in CO-hemoglobin, in which the imidazole moiety constitutes an axial ligand *trans* to the CO. These studies do point out an interesting topological feature. The CO ligand would be proximal to the valerate-threonine-bithiazole side chain (the suspected DNA binding domain) no matter what the relative order of the three remaining ligands. By analogy then, such a geometry would direct the O_2 ligand and any radicals derived therefrom toward the DNA, thus suggesting a possible explanation for the observed selectivity (23) of the DNA damage. This model further predicts that any modifications in bleomycin can lead to repositioning of the ligands around the metal. The potential targeting could then be disrupted either by coordination of O_2 distal to the DNA or by prevention of O_2 binding.

The large size and complexity of bleomycin raises questions as to whether it may serve some direct metabolic role in the microorganism other than its activity as an antibiotic. For example, bleomycin could serve as a divalent metal ion scavenger or ionophore in order to sequester or regulate such ions as copper(II). Alternatively, the high affinity of the Fe(II)–bleomycin complex for O_2 might suggest a possible role as a primitive hemoglobin. Finally, the ability to generate superoxide or hydroxyl radicals could indicate a possible role as a primitive *P*-450 system for the degradation of metabolites.

This investigation establishes Fe(II)–bleomycin–CO as a stable analog of the "active" Fe(II)–bleomycin–O_2 complex and has allowed assignment of the ligands coordinated to the Fe(II) ion. Fluorescence quenching experiments have shown that it readily binds to DNA without effecting significant thymine release, an observation of importance for investigations of bleomycin binding to DNA.

We thank Dr. Gerald Wogan for use of the spectrofluorimeter. This research was in part supported by National Institutes of Health Grants CA-26272 (to N.J.O.) and CA-22614 (to S.M.H.), Stanford Magnetic Resonance Laboratory Research Resource Center Grants NSF-GP-23633 and NIH-RR-00711, and University of California, San Francisco, NMR Research Resource Center Grant NIH-RR-00892. L.O.R. is a Fellow of the Economic Development Administration of Puerto Rico. S.M.H. is an Alfred P. Sloan Fellow, 1975–1979; National Institutes of Health Research Career Development Awardee, 1975–1980; and John Simon Guggenheim Fellow, 1977–1978.

1. Remers, W. A. (1979) *The Chemistry of Antitumor Antibiotics* (John Wiley, New York), Vol. 1, pp. 176–220.
2. Umezawa, H., Maeda, K., Takeuchi, T. & Okami, Y. (1966) *J. Antibiot. Ser. A* **19**, 200–209.
3. Sausville, E. A., Peisach, J. & Horwitz, S. B. (1976) *Biochem. Biophys. Res. Commun.* **73**, 814–822.
4. Lown, J. W. & Sim, S.-K. (1977) *Biochem. Biophys. Res. Commun.* **77**, 1150–1157.
5. Müller, W. E. G., Yamazaki, Z., Breter, H. J. & Zahn, R. K. (1972) *Eur. J. Biochem.* **31**, 518–525.
6. Sausville, E. A., Peisach, J. & Horwitz, S. B. (1978) *Biochemistry* **17**, 2740–2746.
7. Takita, T., Muraoka, Y., Nakatani, T., Fujii, A., Iitaka, Y. & Umezawa, H. (1978) *J. Antibiot.* **31**, 1073–1077.
8. Sugiura, Y. & Kikuchi, T. (1978) *J. Antibiot.* **31**, 1310–1312.
9. Oberley, L. W. & Buettner, G. R. (1979) *FEBS Lett.* **97**, 47–49.
10. Dabrowiak, J. C., Greenaway, F. T., Longo, W. E., Van Husen, M. & Crooke, S. T. (1978) *Biochim. Biophys. Acta* **517**, 517–526.
11. Cass, A. E. G., Galdes, A., Hill, H. A. O. & McClelland, C. E. (1978) *FEBS Lett.* **89**, 187–190.
12. Oppenheimer, N. J. (1979) in *Bleomycin: Chemistry, Biochemistry and Biological Aspects*, ed. Hecht, S. M. (Springer-Verlag, New York), pp. 124–137.
13. Oppenheimer, N. J., Rodriguez, L. O. & Hecht, S. M. (1979) *Biochemistry* **18**, 3439–3445.
14. Iitaka, Y., Nakamura, H., Nakatani, T., Muraoka, Y., Fujii, A., Takita, T. & Umezawa, H. (1978) *J. Antibiot.* **31**, 1070–1072.
15. Chien, M., Grollman, A. P. & Horwitz, S. B. (1977) *Biochemistry* **16**, 3641–3646.
16. Espejo, R. T. & Canelo, E. S. (1968) *Virology* **34**, 738–747.
17. Gupta, R. K. & Redfield, A. G. (1970) *Science* **169**, 1204–1206.
18. Dabrowiak, J. C., Greenaway, F. T. & Grulich, R. (1978) *Biochemistry* **17**, 4090–4096.
19. Sugiura, Y. (1979) *Biochem. Biophys. Res. Commun.* **87**, 643–648.
20. Nakayama, Y., Kunishima, M., Omoto, S., Takita, T. & Umezawa, H. (1973) *J. Antibiot.* **26**, 400–402.
21. Hori, M. (1979) in *Bleomycin: Chemistry, Biochemistry and Biological Aspects*, ed. Hecht, S. M. (Springer-Verlag, New York), pp. 195–206.
22. Crippen, G. M. (1977) *J. Comp. Phys.* **24**, 96–107.
23. D'Andrea, A. D. & Haseltine, W. A. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3608–3612.

US005726023A

United States Patent [19]

Cheever et al.

[11] Patent Number: 5,726,023

[45] Date of Patent: Mar. 10, 1998

[54] **IMMUNE REACTIVITY TO HER-2/NEU PROTEIN FOR DIAGNOSIS AND TREATMENT OF MALIGNANCIES IN WHICH THE HER-2/NEU ONCOGENE IS ASSOCIATED**

[75] Inventors: **Martin A. Cheever**, Mercer Island; **Mary L. Disis**, Renton, both of Wash.

[73] Assignee: **University of Washington**, Seattle, Wash.

[21] Appl. No.: **467,083**

[22] Filed: **Jun. 6, 1995**

Related U.S. Application Data

[63] Continuation of Ser. No. 414,417, Mar. 31, 1995, which is a continuation-in-part of Ser. No. 106,112, Aug. 12, 1993, abandoned, which is a continuation-in-part of Ser. No. 33,644, Mar. 17, 1993, abandoned.

[51] **Int. Cl.**[6] **G01N 33/536**; G01N 33/541; G01N 33/566; C07K 16/30

[52] **U.S. Cl.** **435/7.1**; 435/7.5; 435/7.9; 435/7.91; 435/7.92; 436/501; 436/536; 436/538; 436/540; 436/542; 436/547; 530/387.7; 530/389.7

[58] **Field of Search** 424/138.1, 139.1, 424/155.1, 156.1, 520; 435/7.1, 7.5, 7.23, 7.9, 7.91–7.95, 188; 436/501, 512, 542, 548; 530/387.7, 388.8, 389.7, 391.3, 391.5, 391.9

[56] **References Cited**

U.S. PATENT DOCUMENTS

3,766,162	10/1973	Spector	260/112 R
3,791,932	2/1974	Schuurs et al.	195/103.5 R
3,817,837	6/1974	Rubenstein et al.	195/103.5 R
3,850,752	11/1974	Schuurs et al.	195/103.5 R
3,901,654	8/1975	Gross	23/230 B
3,935,074	1/1976	Rubenstein et al.	195/103.5 R
3,984,533	10/1976	Uzgiris	424/12
3,996,345	12/1976	Ullman et al.	424/12
4,034,074	7/1977	Miles	424/1
4,098,876	7/1978	Piasio et al.	424/1
4,233,402	11/1980	Maggio et al.	435/7
4,452,901	6/1984	Gordon et al.	436/506
5,320,947	6/1994	Cheever .	
5,401,638	3/1995	Carney .	

FOREIGN PATENT DOCUMENTS

WO 91/02062 2/1991 WIPO .

OTHER PUBLICATIONS

Weiner Nature 339:230–231 1989.

Ben-Mahrez Br. J. Cancer 57:529–534 1988.

Ben-Mahrez Int. J. Cancer 46:35–38, 1990.

Wisdom Clin Chem 2218 1243–1255, 1976.

Alper et al., "The Presence of c-erbB-2 Gene Product-related Protein in Culture medium Conditioned by Breast Cancer Cell Line SK-BR-3," *Cell Growth & Differentiation* 1:591–599, 1990.

Bargann et al., "The neu oncogene encodes an epidermal growth factor receptor-related protein," *Nature* 319:226–230, 1986.

Bishop and Orosz, "Limiting Dilution Analysis For Alloreactive, TCGF-Secretory T Cells. Two Related LDA Methods That Discriminate Between Unstimulated Precursor T Cells and In Vivo-Alloactivated T Cells," *Transplantation* 47(4):671–677, 1989.

Bowen-Pope et al., "Production of platelet-derived growth factor-like molecules and reduced expression of platelet-derived growth factor receptors accompany transformation by a wide spectrum of agents," *Proc. Natl. Acad. Sci. USA* 81:2396–2400, 1984.

Brooks et al., "Human lymphocyte markers defined by antibodies derived from somatic cell hybrids," *Clin. exp. Immunol.* 39:477–485, 1980.

Brown et al., "Protein Antigens of Normal and Malignant Human Cells Identified by Immunoprecipitation with Monoclonal Antibodies," *Journal of Biological Chemistry* 255(11):4980–4983, 1980.

Burnette et al., "Western Blotting:Electropheretic Transfer of Proteins from Sodium Dodecyl Sulfate-Palacrylamide Gels to Unmodified Nitrocellulose and Radiographic Detection with Antibody and Radioiodinated Protein A," *Analytical Biochemistry* 112:195–203, 1981.

Dhut et al, "BCR-ABL and BCR Proteins: Biochemical Characterization and Localization," *Leukemia* 4(11):745–750, 1990.

Falk et al., "Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules," *Nature* 351:290–296, 1991.

Feller and de la Cruz, "Identifying antigenic T-cell sites," *Nature* 349:720–721, 1991.

Iglehart et al., "Increased erbB-2 Gene Copies and Expression in Multiple Stages of Breast Cancer," *Cancer Research* 50:6701–6707, 1990.

Ioannides et al., "T-Cell Recognition of Oncogene Products: A New Strategy for Immunotherapy," *Molecular Carcinogenesis* 6:77–82, 1992.

Kallioniemi et al., "ERBBamplification in breast cancer analyzed by fluorescence in situ hybridization," *Proc. Natl. Acad. Sci.* 89:5321–5325, 1992.

(List continued on next page.)

Primary Examiner—Lila Feisee
Assistant Examiner—Nancy A. Johnson
Attorney, Agent, or Firm—Seed and Berry LLP

[57] **ABSTRACT**

Methods for the detection, monitoring and treatment of malignancies in which the HER-2/neu oncogene is associated are disclosed. Detection of specific T cell activation (e.g., by measuring the proliferation of T cells) in response to in vitro exposure to the HER-2/neu protein, or detection of immunocomplexes formed between the HER-2/neu protein and antibodies in body fluid, allows the diagnosis of the presence of a malignancy in which the HER-2/neu oncogene is associated. The present invention also discloses methods and compositions, including peptides, for treating such malignancies.

20 Claims, 21 Drawing Sheets

OTHER PUBLICATIONS

Kerns et al., "c-erbB-2 Expression In Breast Cancer Detected by Immunoblotting and Immunohistochemistry," *Journal of Histochemistry and Cytochemistry* 38(12):1823-1830, 1990.

Laemmli, U., "Cleavage of Structural Proteins during the assembly of the Head of Bacteriophage T4," *Nature* 227:680-685, 1970.

Leitzel et al., "Elevated Soluble c-erbB2 Antigen Levels in the Serum and Effusions of a Proportion of Breast Cancer Patients," *Journal of Clinical Oncology* 10(9):1436-1443, 1992.

Lippman, M., "Potential Contributions of Breast Cancer Biology to Management of Breast Cancer," *Advances in Oncology* 8(3):26-28, 1992.

Maguire and Greene, "The neu (c-erbB-2) Oncogene," *Seminars in Oncology* 16(2):148-155, 1989.

Margalit et al., "Prediction Of Immunodominant Helper T Cell Antigenic Sites From The Primary Sequence," *Journal of Immunology* 138(7):2213-2229, 1987.

McKenzie et al., "Induction of Antitumor Immunity by Immunization with a Vaccinia Virus Vector Expressing and Oncogene-encoded Product," in *Vaccines 88*, Cold Spring Harbor Laboratory, 1988, pp. 19-23.

Mietzner et al., "Purification and Characterization of the Major Iron-Regulated Protein Expressed By Pathogenic Neisseriae," *Journal of Experimental Medicine* 165:1041-1057, 1987.

Mori et al., "In vitro and in vivo Release of Soluble erbB-2 Protein from Human Carcinoma Cells," *Japanese Journal of Cancer Research* 81:489-494, 1990.

Paik et al., "Pathologic Findings From the National Surgical Adjuvant Breast and Bowel Project: Prognostic Significance of erbB-2 Protein Overexpression in Primary Breast Cancer," *Journal of Clinical Oncology* 8(1):103-112, 1990.

Parker et al., "Sequence Motifs Important For Peptide Binding To The Human MHC Class I Molecule, HLA-A2," *Journal of Immunology* 149(11):3580-3587, 1992.

Press et al., "Expression of the HER-2/neu proto-oncogene in normal human adult and fetal tissues," *Oncogene* 5:953-962, 1990.

Raines and Ross, "Platelet-derived Growth Factor. I. High Yields Purification and Evidence for Multiple Forms," *Journal of Biological Chemistry* 257(9):5154-5159, 1982.

Riberdy and Cresswell, "The Antigen-Processing Mutant T2 Suggests a Role for MHC-Linked Genes in Class II Antigen Presentation," *Journal of Immunology* 148(8):2586-2590, 1992.

Rothbard and Taylor, "A sequence pattern common to T cell epitopes," *EMBO Journal* 7(1) 93-100, 1988.

Spies et al., "A gene in the human major histocompatibility complex class II region controlling the class I antigen presentation pathway," *Nature* 348:744-747, 1990.

Stern et al., "Oncogenic Activation of $p185^{neu}$ Stimulates Tyrosine Phosphorylation In Vivo," *Molecular and Cellular Biology* 8(9):3969-3973, 1988.

Trowsdale et al., "Sequence encoded in the class II region of the MHC related to the 'ABC' superfamily of transporters," *Nature* 348:741-744, 1990.

Wide, L., "Solid Phase Antigen-Antibody Systems," *in Radioimmunoassay Methods*, Kirkham and Hunter (eds.), 1973, pp. 405-412.

Zabrecky et al., "The Extracellular Domain of p185/neu Is Released from the Surface of Human Breast Carcinoma Cells, SK-BR-3," *Journal of Biological Chemistry* 266(3):1716-1720, 1991.

```
    5   10   15   20   25   30   35   40   45   50   55   60   65   70   75
MELAALCRWGLLLALLPPGAASTQVCTGTDMKLRLPASPETHLDMLRHLYQGCQVVQGNLELTYLPTNASLSFLQ

   80   85   90   95  100  105  110  115  120  125  130  135  140  145  150
DIQEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDNYALAVLDNGDPLNNTTPVTGASPGGLRELQLRSLTEILK

  155  160  165  170  175  180  185  190  195  200  205  210  215  220  225
GGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLIDTNRSRACHPCSPMCKGSRCWGESSEDCQSLTRTVCAGGCA

  230  235  240  245  250  255  260  265  270  275  280  285  290  295  300
RCKGPLPTDCCHEQCAAGCTGPKHSDCLACLHFNHSGICELHCPALVTYNTDTFESMPNPEGRYTFGASCVTACP

  305  310  315  320  325  330  335  340  345  350  355  360  365  370  375
YNYLSTDVGSCTLVCPLHNQEVTAEDGTQRCEKCSKPCARVCYGLGMEHLREVRAVTSANIQEFAGCKKIFGSLA

  380  385  390  395  400  405  410  415  420  425  430  435  440  445  450
FLPESFDGDPASNTAPLQPEQLQVFETLEEITGYLYISAWPDSLPDLSVFQNLQVIRGRILHNGAYSLTLQGLGI

  455  460  465  470  475  480  485  490  495  500  505  510  515  520  525
SWLGLRSLRELGSGLALIHHNTHLCFVHTVPWDQLFRNPHQALLHTANRPEDECVGEGLACHQLCARCHCWGPGP

  530  535  540  545  550  555  560  565  570  575  580  585  590  595  600
TQCVNCSQFLRGQECVEECRVLQGLPREYVNARHCLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARC

  605  610  615  620  625  630  635  640  645  650  655  660  665  670  675
PSGVKPDLSYMPIWKFPDEEGACQPCPINCTHSCVDLDDKGCPAEQRASPLTSIISAVVGILLVVVLGVVFGILI

  680  685  690  695  700  705  710  715  720  725  730  735  740  745  750
KRRQQKIRKYTMRRLLQETELVEPLTPSGAMPNQAQMRILKETELRKVKVLGSGAFGTVYKGIWIPDGENVKIPV

  755  760  765  770  775  780  785  790  795  800  805  810  815  820  825
AIKVLRENTSPKANKEILDEAYVMAGVGSPYVSRLLGICLTSTVQLVTQLMPYGCLLDHVRENRGRLGSQDLLNW

  830  835  840  845  850  855  860  865  870  875  880  885  890  895  900
CMQIAKGMSYLEDVRLVHRDLAARNVLVKSPNHVKITDFGLARLLDIDETEYHADGGKVPIKWMALESILRRRFT

  905  910  915  920  925  930  935  940  945  950  955  960  965  970  975
HQSDVWSYGVTVWELMTFGAKPYDGIPAREIPDLLEKGERLPQPPICTIDVYMIMVKCWMIDSECRPRFRELVSE

  980  985  990  995 1000 1005 1010 1015 1020 1025 1030 1035 1040 1045 1050
FSRMARDPQRFVVIQNEDLGPASPLDSTFYRSLLEDDDMGDLVDAEEYLVPQQGFFCPDPAPGAGGMVHHRHRSS

 1055 1060 1065 1070 1075 1080 1085 1090 1095 1100 1105 1110 1115 1120 1125
STRSGGGDLTLGLEPSEEEAPRSPLAPSEGAGSDVFDGDLGMGAAKGLQSLPTHDPSPLQRYSEDPTVPLPSETD

 1130 1135 1140 1145 1150 1155 1160 1165 1170 1175 1180 1185 1190 1195 1200
GYVAPLTCSPQPEYVNQPDVRPQPPSPREGPLPAARPAGATLERPKTLSPGKNGVVKDVFAFGGAVENPEYLTPQ

 1205 1210 1215 1220 1225 1230 1235 1240 1245 1250 1255
GGAAPQPHPPPAFSPAFDNLYYWDQDPPERGAPPSTFKGTPTAENPEYLGLDVPV
```

Fig. 1

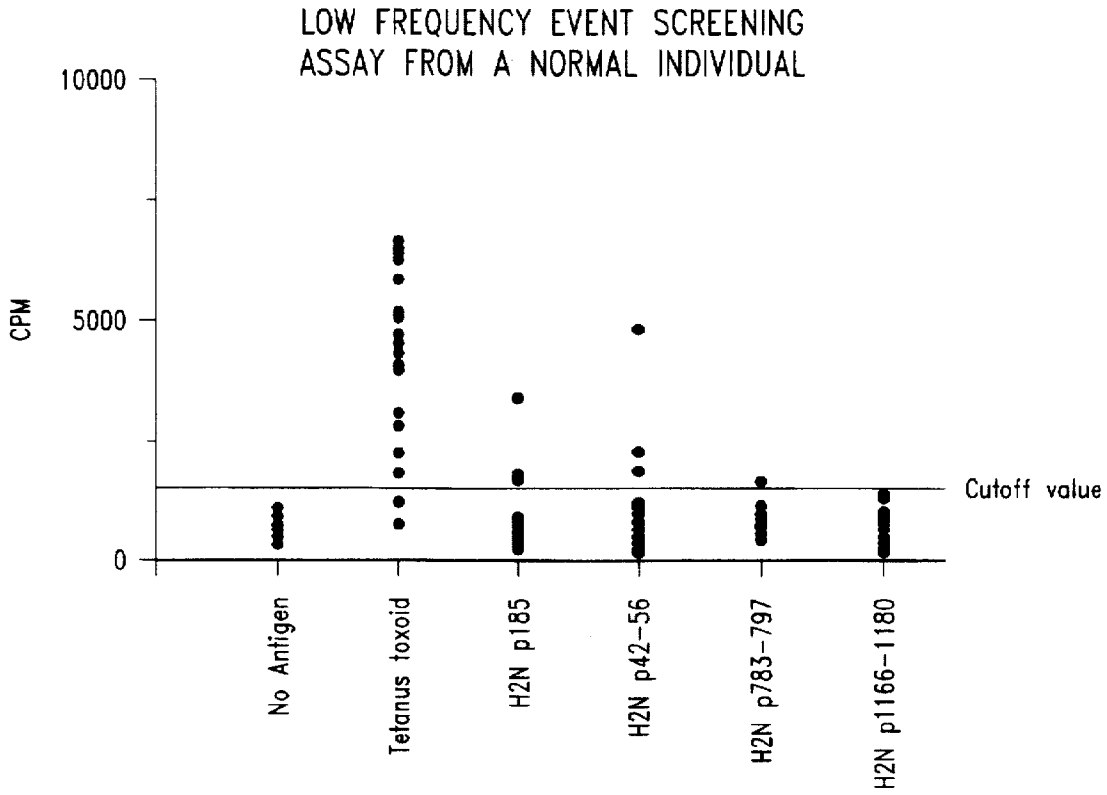

LOW FREQUENCY EVENT SCREENING
ASSAY FROM A NORMAL INDIVIDUAL
10000
5000
0
CPM
Cutoff value
No Antigen
Tetanus toxoid
H2N p185
H2N p42-56
H2N p783-797
H2N p1166-1180

Fig. 2

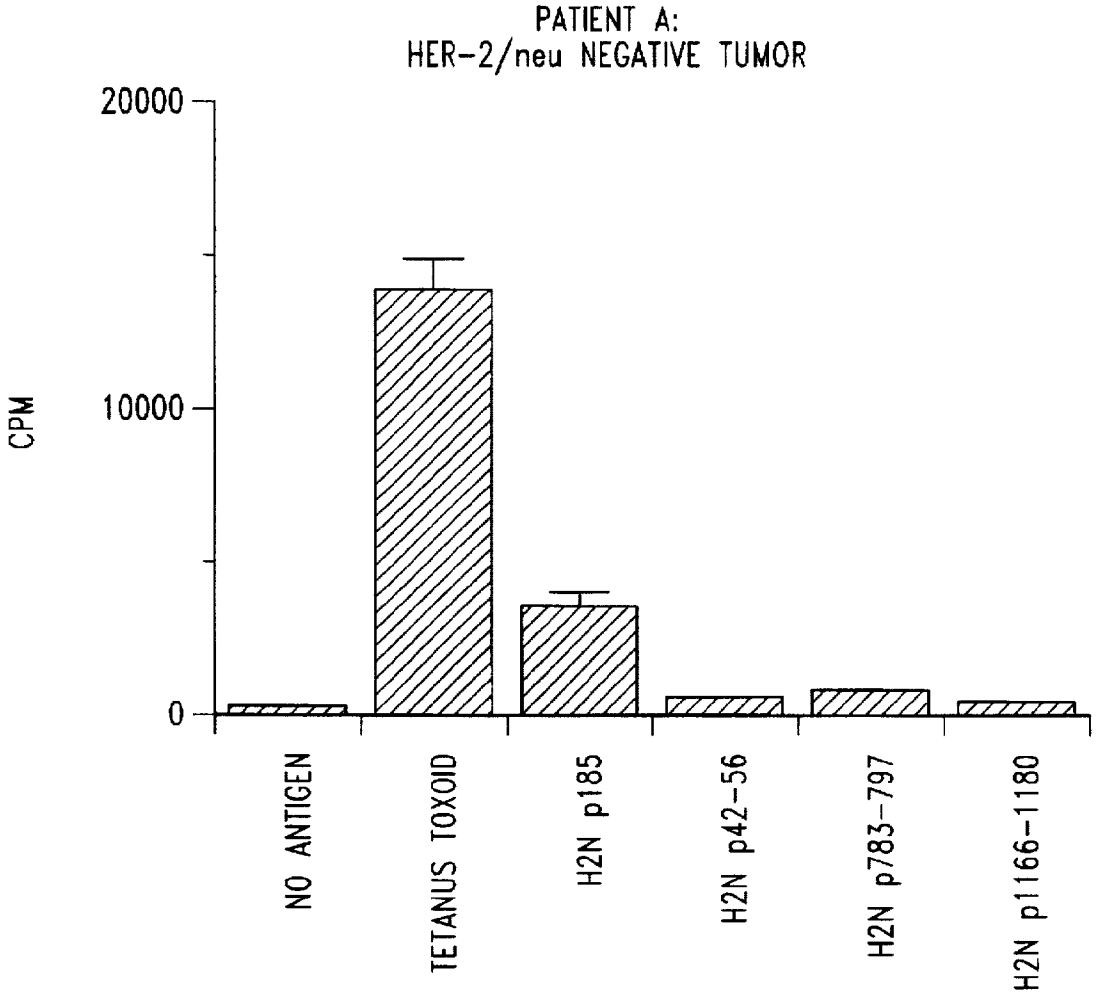

PATIENT A:
HER-2/neu NEGATIVE TUMOR
20000
10000
0
CPM
NO ANTIGEN
TETANUS TOXOID
H2N p185
H2N p42-56
H2N p783-797
H2N p1166-1180

Fig. 3A

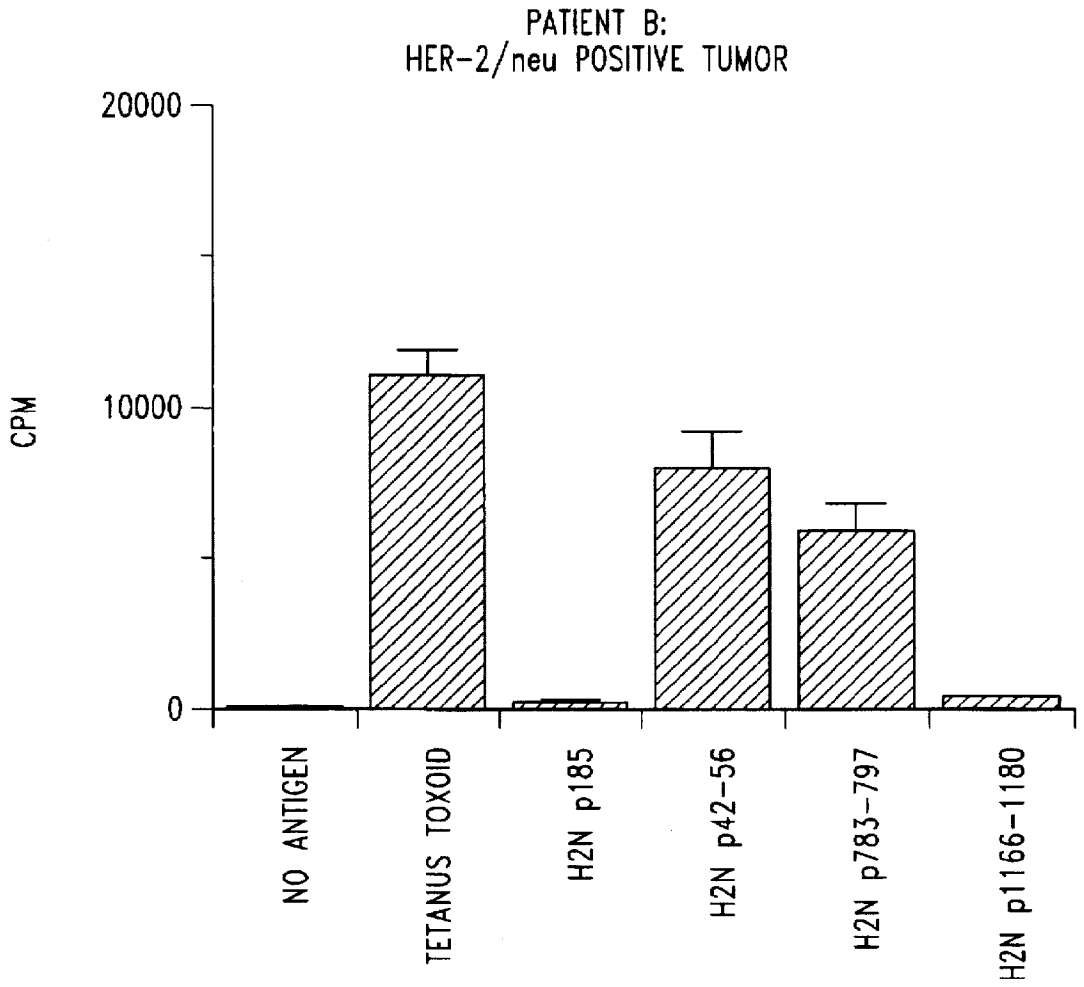

PATIENT B:
HER-2/neu POSITIVE TUMOR
CPM
20000
10000
0
NO ANTIGEN
TETANUS TOXOID
H2N p185
H2N p42-56
H2N p783-797
H2N p1166-1180

Fig. 3B

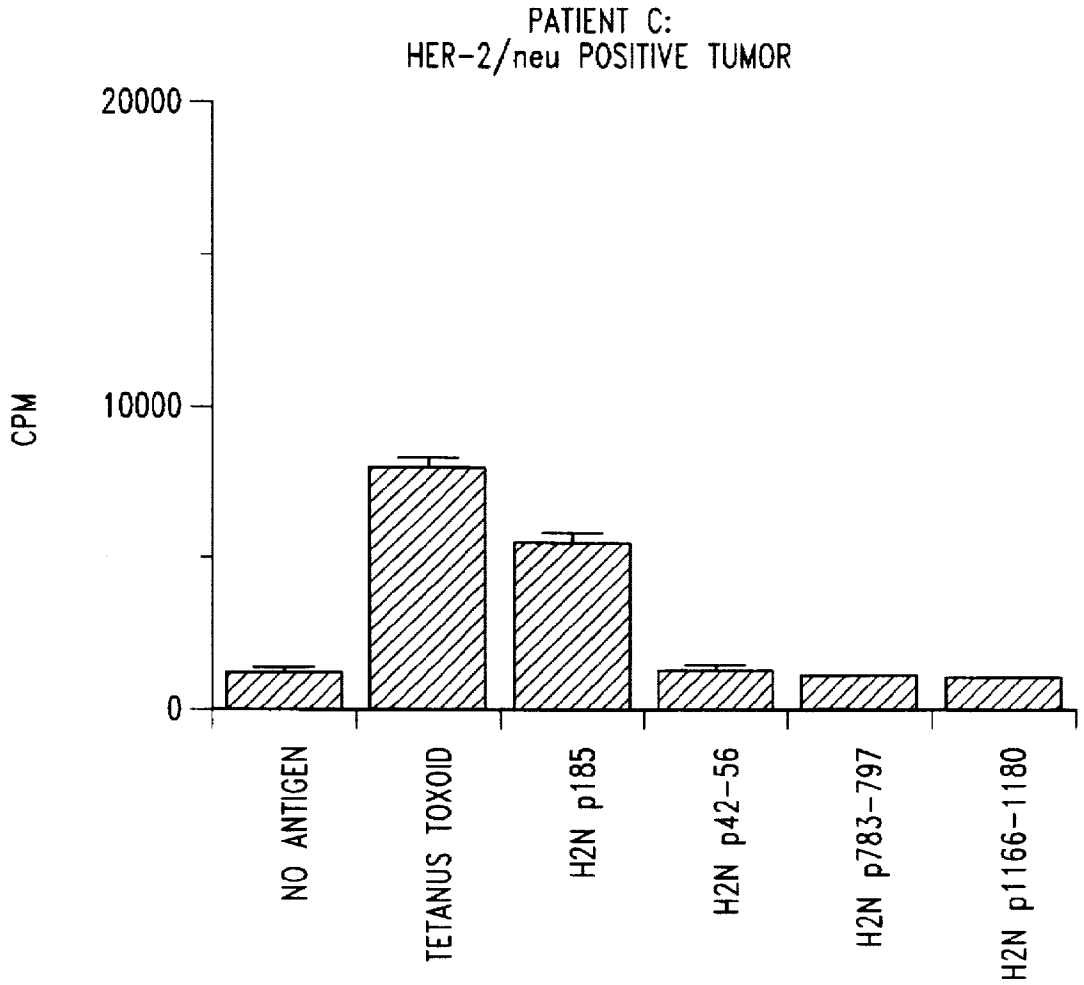

PATIENT C:
HER-2/neu POSITIVE TUMOR
20000
10000
0
CPM
NO ANTIGEN
TETANUS TOXOID
H2N p185
H2N p42-56
H2N p783-797
H2N p1166-1180

Fig. 3C

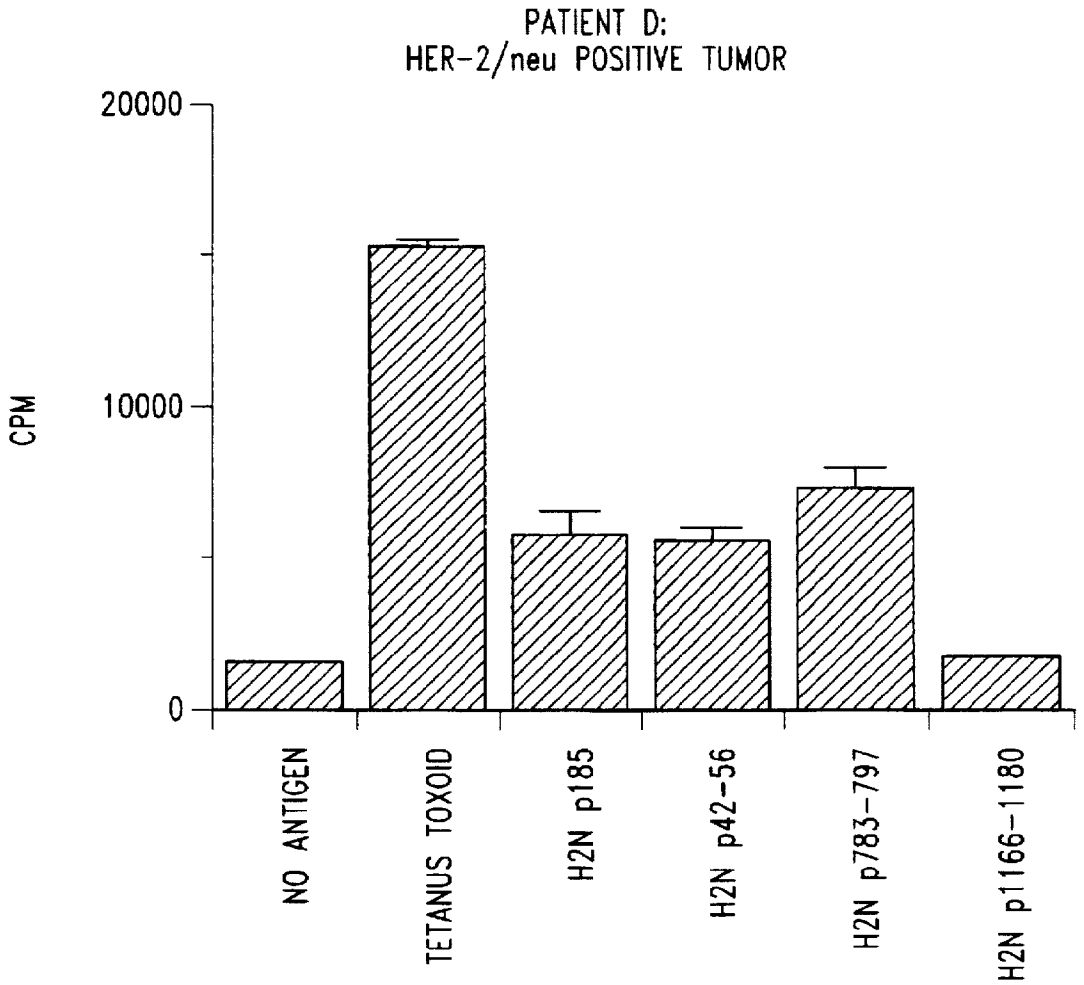

PATIENT D:
HER-2/neu POSITIVE TUMOR
CPM
20000
10000
0
NO ANTIGEN
TETANUS TOXOID
H2N p185
H2N p42-56
H2N p783-797
H2N p1166-1180

Fig. 3D

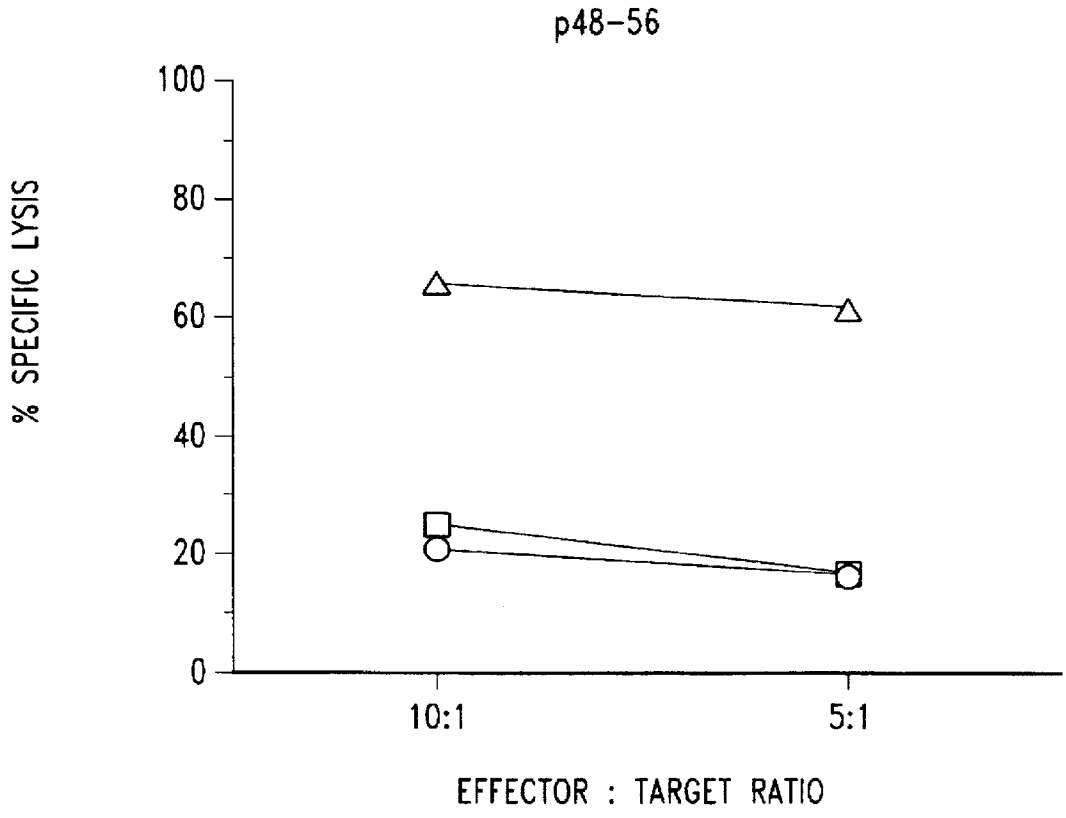

p48-56
100
80
60
40
20
0
% SPECIFIC LYSIS
10:1
5:1
EFFECTOR : TARGET RATIO

Fig. 4A

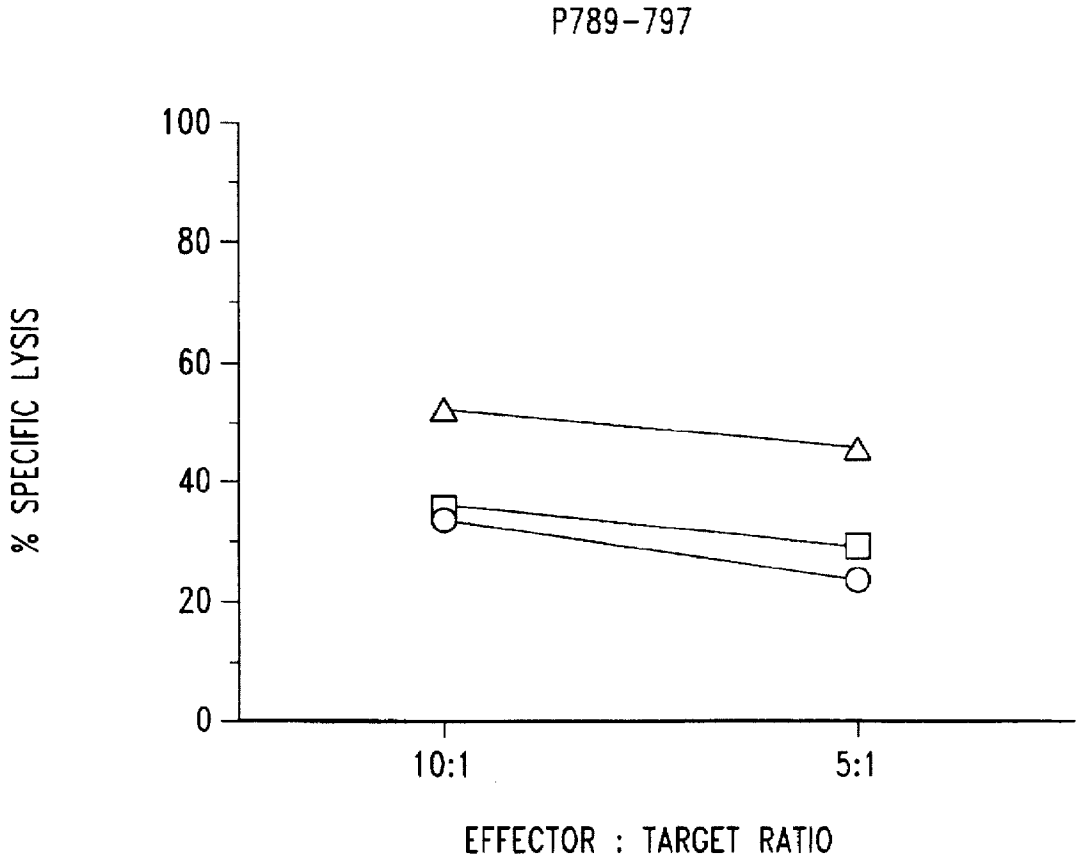

P789-797
100
80
60
40
20
0
% SPECIFIC LYSIS
10:1
5:1
EFFECTOR : TARGET RATIO

Fig. 4B

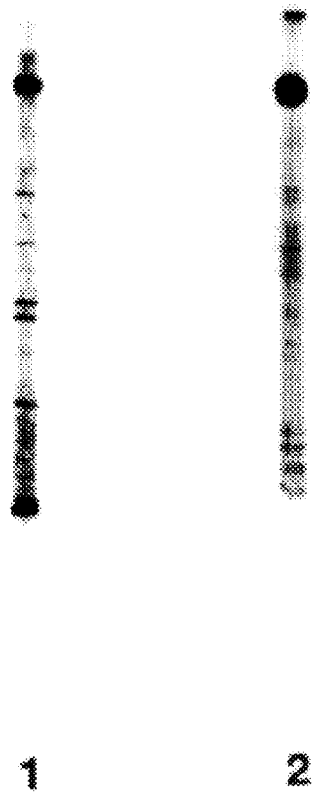


FIGURE 5

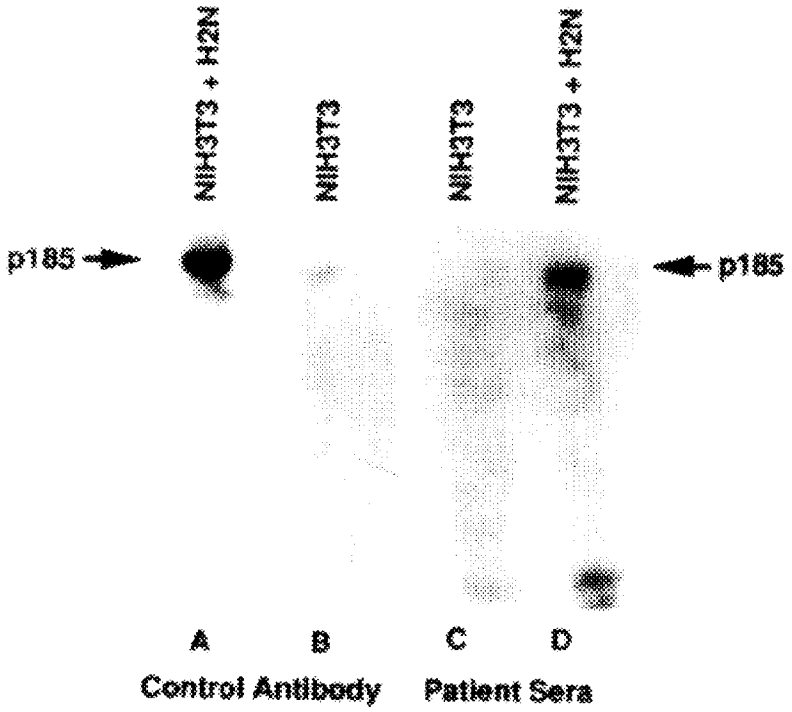

NIH3T3 + H2N
NIH3T3
NIH3T3
NIH3T3 + H2N
p185
p185
A
B
C
D
Control Antibody
Patient Sera

FIGURE 6

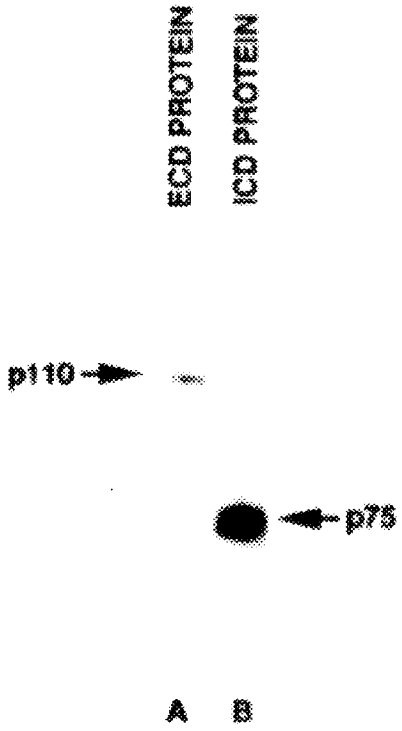


FIGURE 7

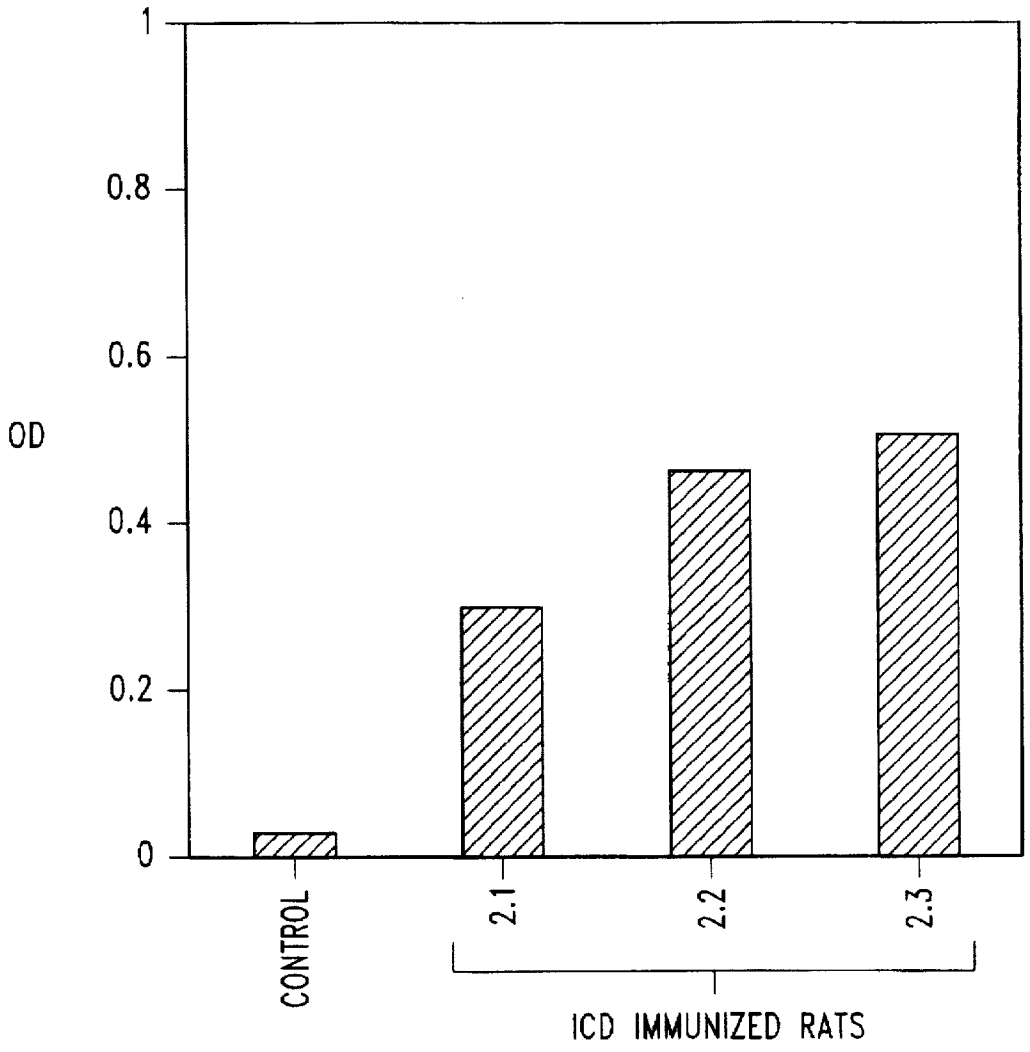


Fig. 8

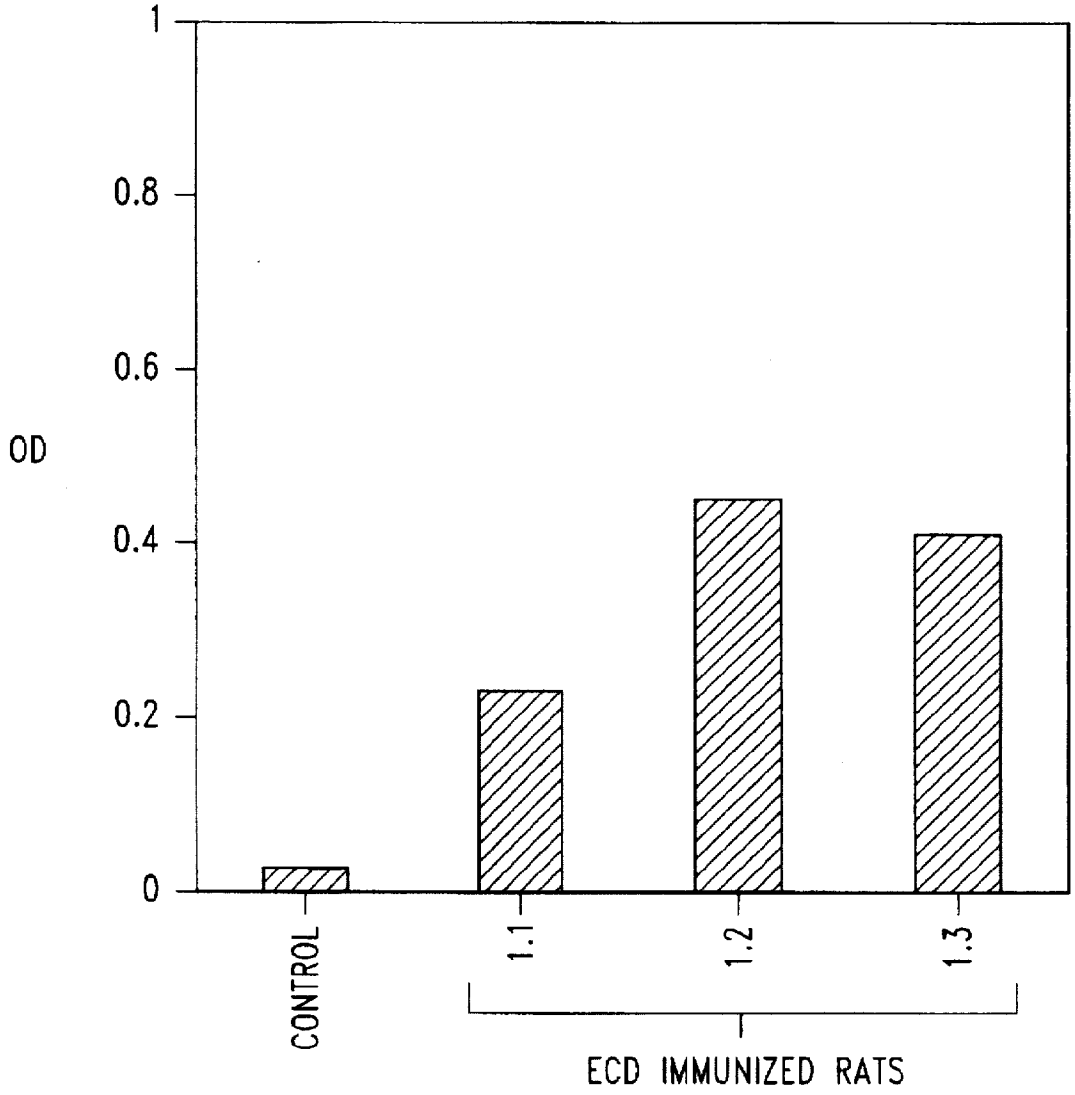


Fig. 9

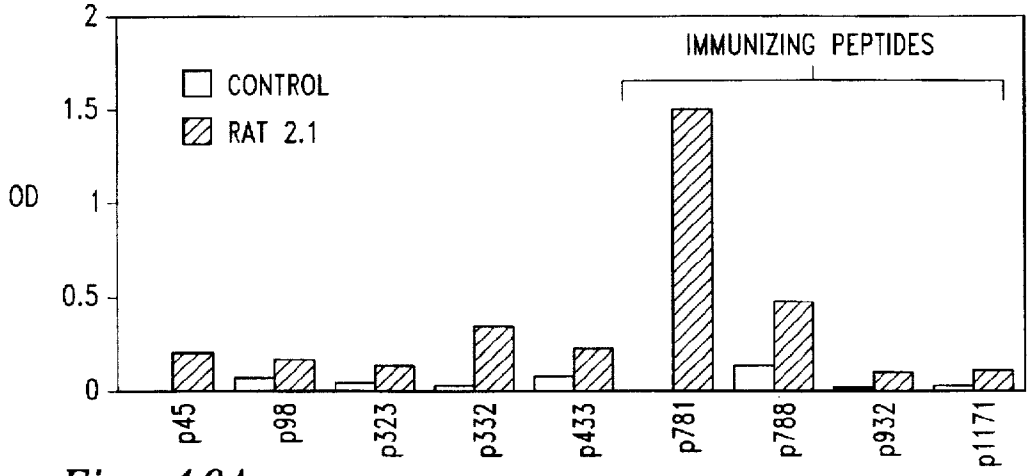


Fig. 10A

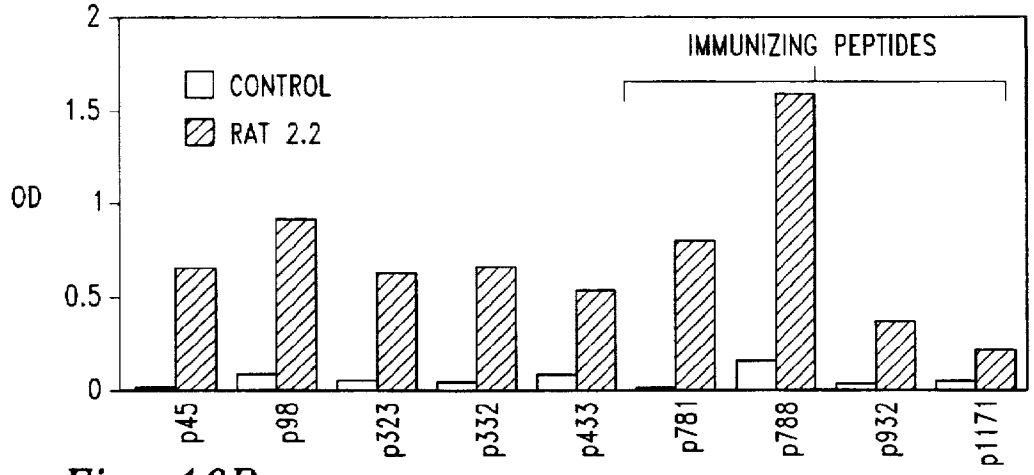


Fig. 10B

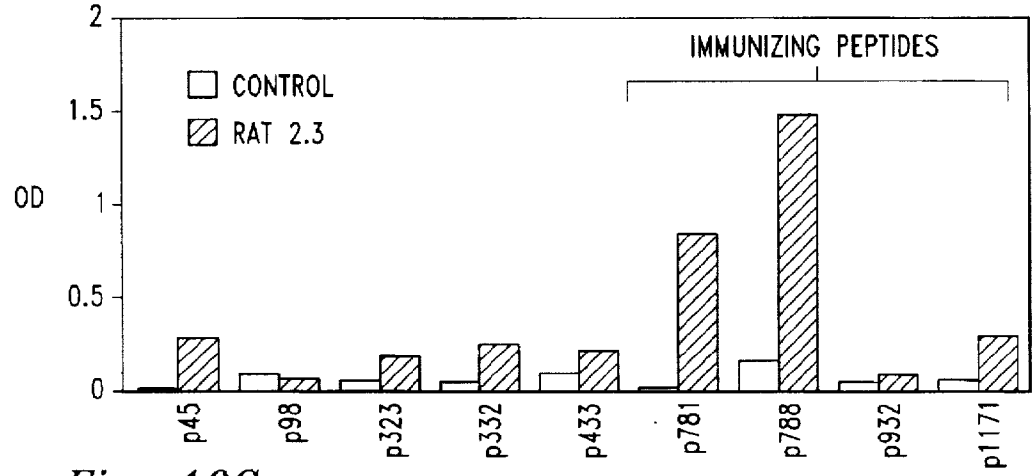


Fig. 10C

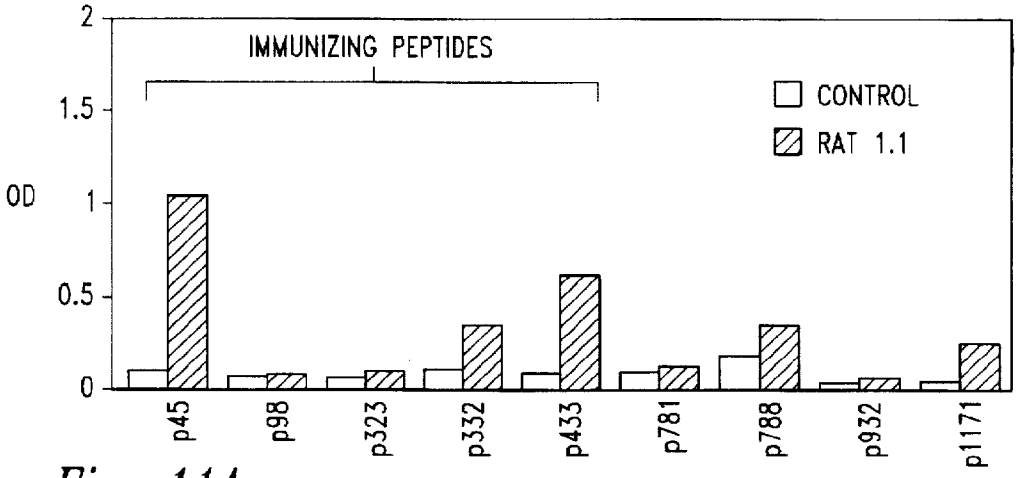


Fig. 11A

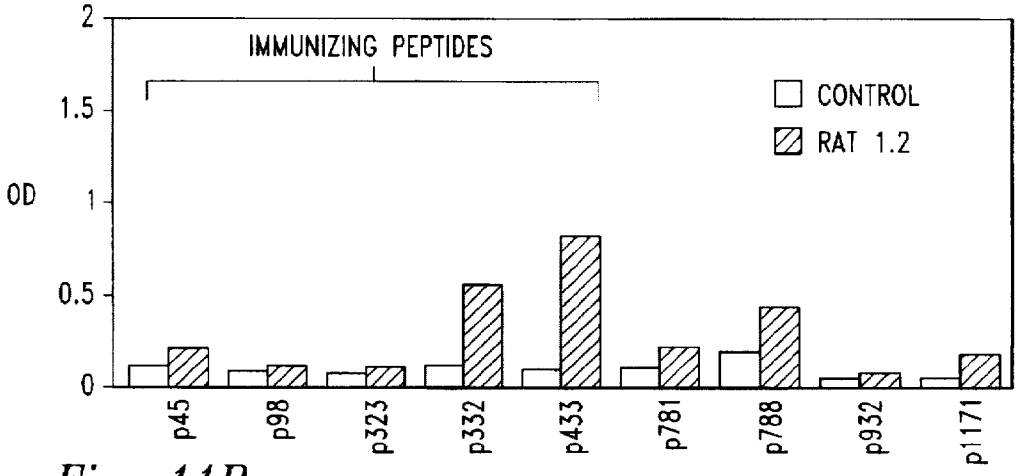


Fig. 11B

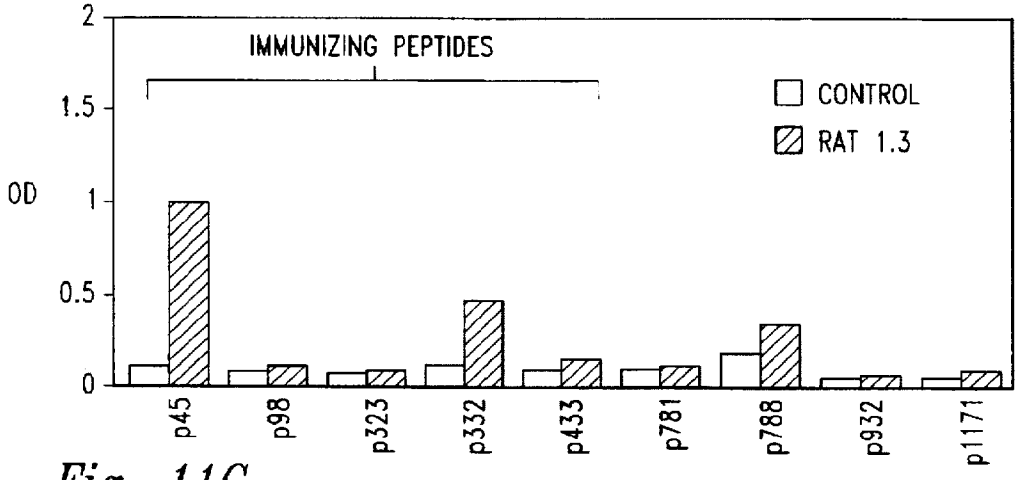


Fig. 11C

FIG. 12A

FIG. 12B

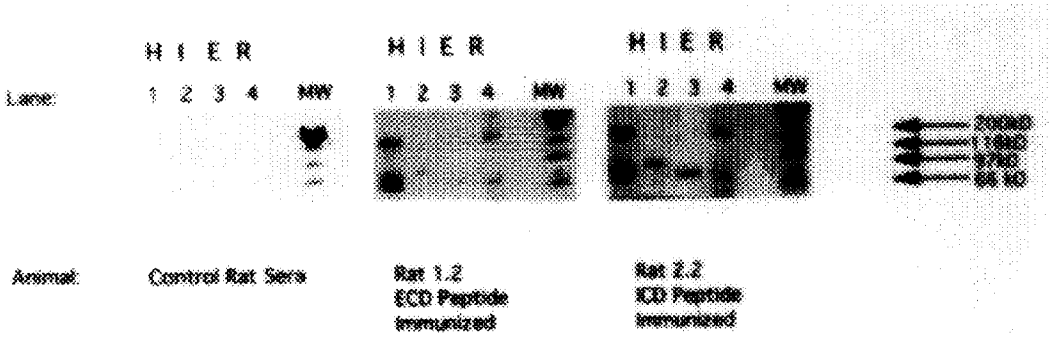

H I E R
H I E R
H I E R
Lane:
1 2 3 4 MW
1 2 3 4 MW
1 2 3 4 MW
Animal:
Control Rat Sera
Rat 1.2
ECD Peptide
Immunized
Rat 2.2
ICD Peptide
Immunized

FIG. 13

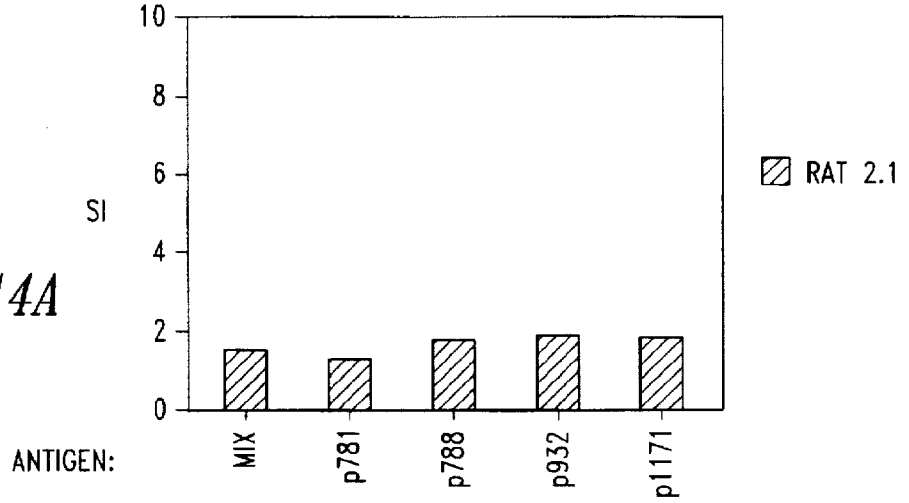


Fig. 14A

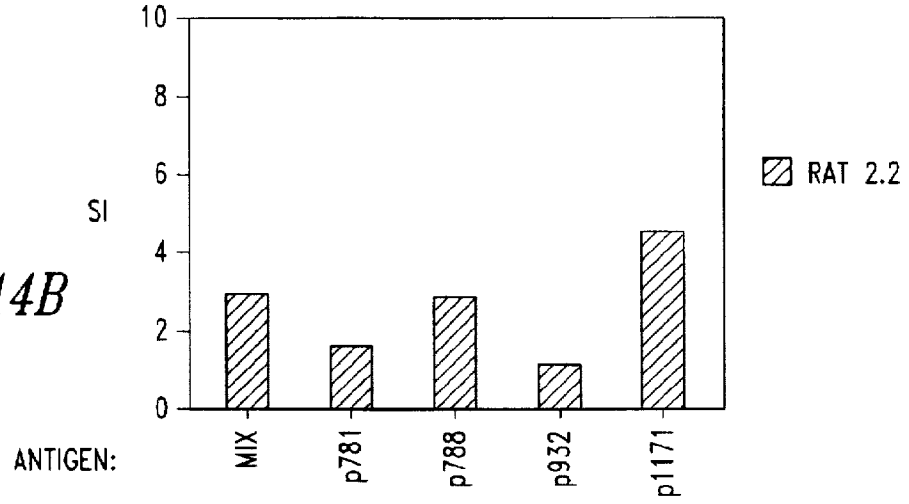


Fig. 14B

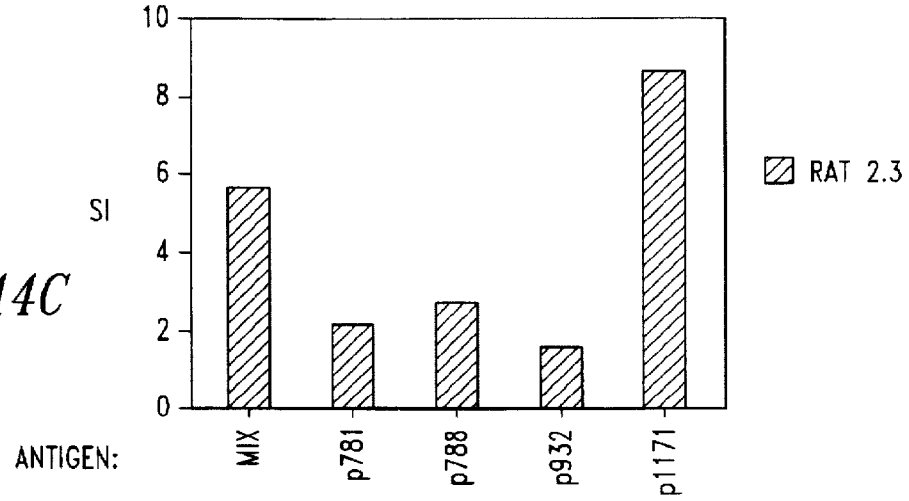


Fig. 14C

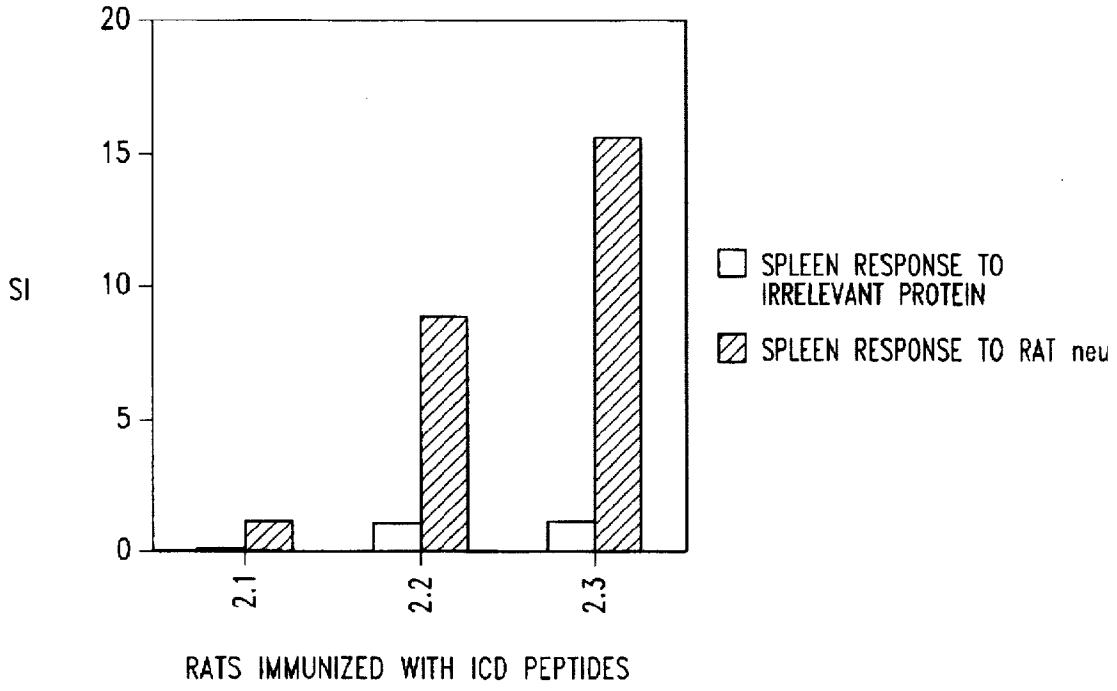

20
15
10
5
0
SI
2.1
2.2
2.3
RATS IMMUNIZED WITH ICD PEPTIDES
SPLEEN RESPONSE TO IRRELEVANT PROTEIN
SPLEEN RESPONSE TO RAT neu

Fig. 15

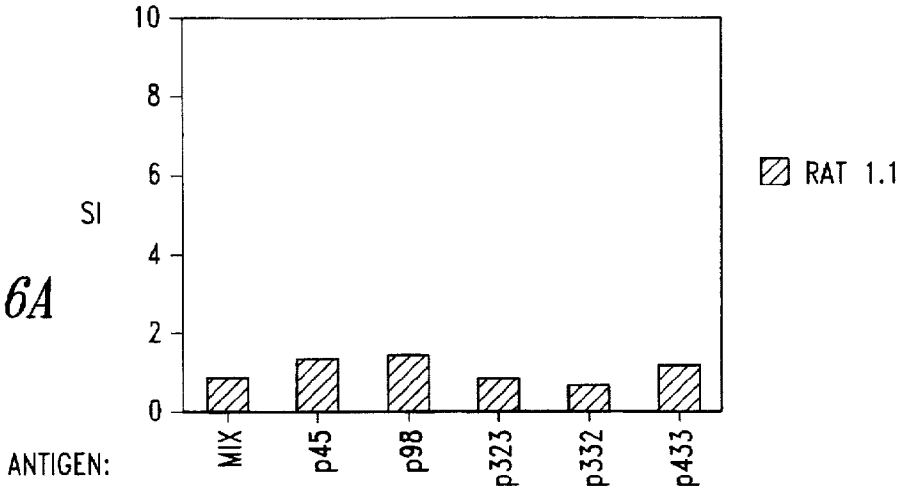


Fig. 16A

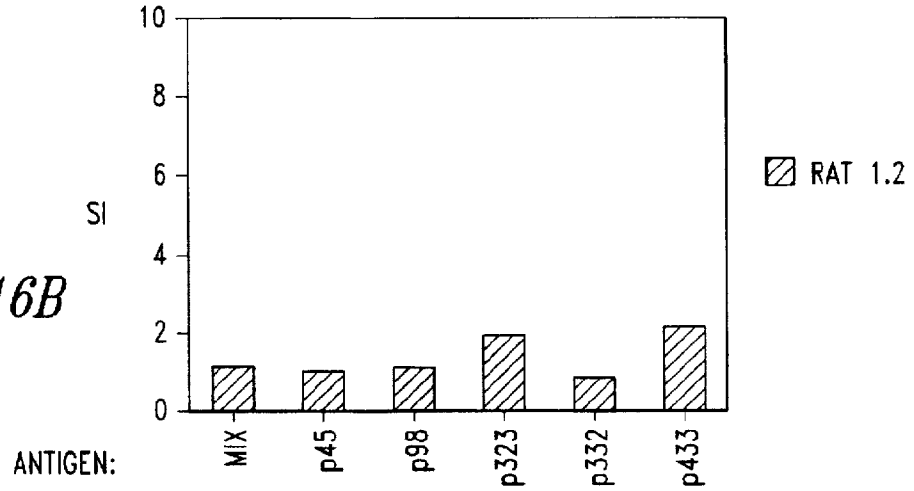


Fig. 16B

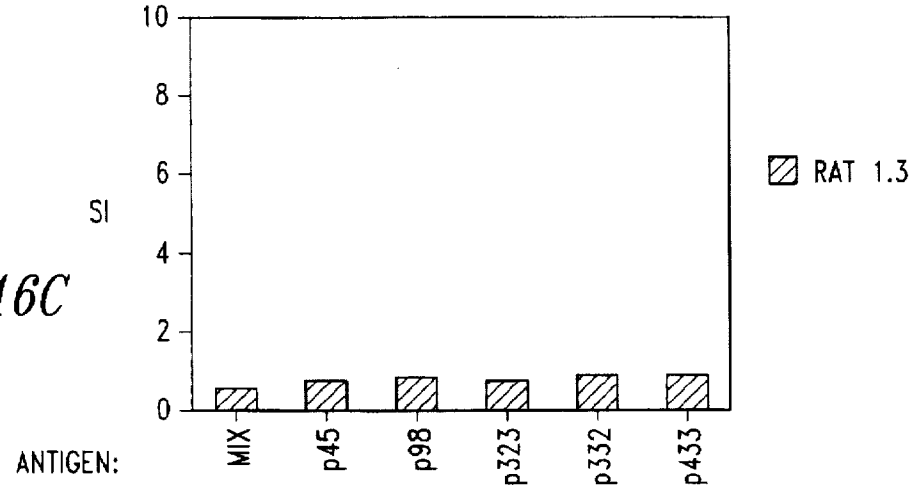


Fig. 16C

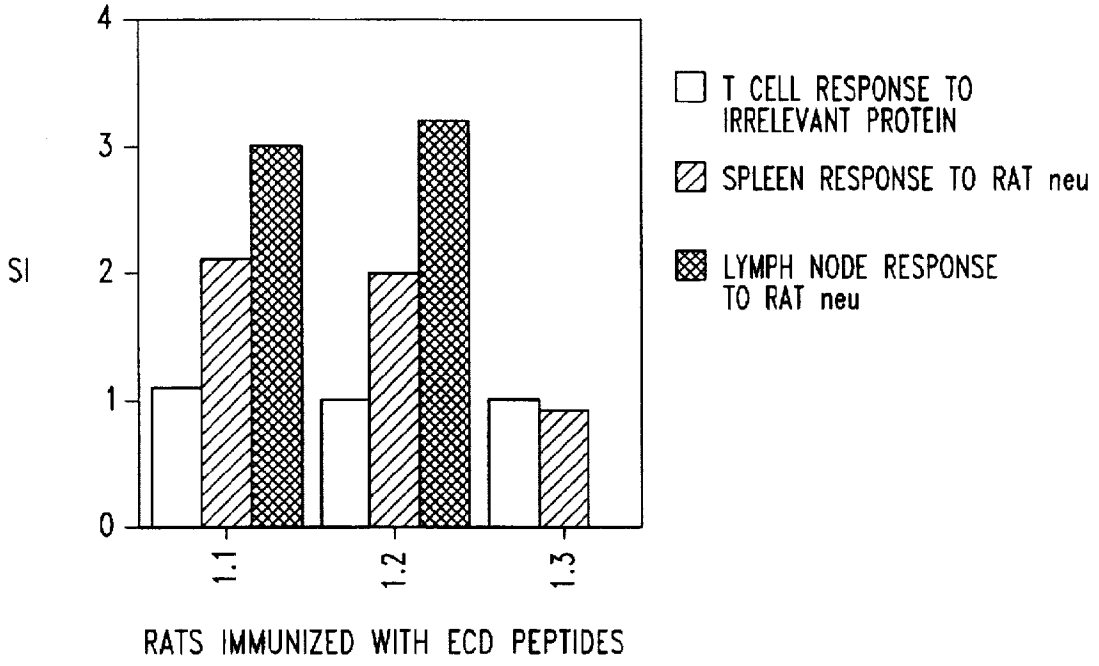

4
3
2
1
0
SI
1.1
1.2
1.3
RATS IMMUNIZED WITH ECD PEPTIDES
T CELL RESPONSE TO IRRELEVANT PROTEIN
SPLEEN RESPONSE TO RAT neu
LYMPH NODE RESPONSE TO RAT neu

Fig. 17

IMMUNE REACTIVITY TO HER-2/NEU PROTEIN FOR DIAGNOSIS AND TREATMENT OF MALIGNANCIES IN WHICH THE HER-2/NEU ONCOGENE IS ASSOCIATED

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 08/414,417, filed Mar. 31, 1995, pending; which application is a continuation-in-part application to Ser. No. 08/106,112, filed Aug. 12, 1993, abandoned; which application is a continuation-in-part application to Ser. No. 08/033,644, filed Mar. 17, 1993, abandoned.

TECHNICAL FIELD

The present invention is generally directed toward the detection, monitoring, and treatment of malignancies, in which the HER-2/neu oncogene associated, through the use of a cancer patient's own immune reactivity to the HER-2/neu protein expressed by the HER-2/neu gene.

BACKGROUND OF THE INVENTION

Despite enormous investments of financial and human resources, cancer remains one of the major causes of death. For example, cancer is the leading cause of death in women between the ages of 35 and 74. Breast cancer is the most common malignancy in women and the incidence for developing breast cancer is on the rise. One in nine women will be diagnosed with the disease. Standard approaches to cure breast cancer have centered around a combination of surgery, radiation and chemotherapy. These approaches have resulted in some dramatic successes in certain malignancies. However, breast cancer is most often incurable, when diagnosed beyond a certain stage. Alternative approaches to early diagnosis and therapy are necessary.

A common characteristic of malignancies is uncontrolled cell growth. Cancer cells appear to have undergone a process of transformation from the normal phenotype to a malignant phenotype capable of autonomous growth. Amplification and overexpression of somatic cell genes is considered to be a common primary event that results in the transformation of normal cells to malignant cells. The malignant phenotypic characteristics encoded by the oncogenic genes are passed on during cell division to the progeny of the transformed cells.

Ongoing research involving oncogenes has identified at least forty oncogenes operative in malignant cells and responsible for, or associated with, transformation. Oncogenes have been classified into different groups based on the putative function or location of their gene products (such as the protein expressed by the oncogene).

Oncogenes are believed to be essential for certain aspects of normal cellular physiology. In this regard, the HER-2/neu oncogene is a member of the tyrosine protein kinase family of oncogenes and shares a high degree of homology with the epidermal growth factor receptor. HER-2/neu presumably plays a role in cell growth and/or differentiation. HER-2/neu appears to induce malignancies through quantitative mechanisms that result from increased or deregulated expression of an essentially normal gene product.

HER-2/neu (p185) is the protein product of the HER-2/neu oncogene. The HER-2/neu gene is amplified and the HER-2/neu protein is overexpressed in a variety of cancers including breast, ovarian, colon, lung and prostate cancer.

HER-2/neu is related to malignant transformation. It is found in 50% –60% of ductal in situ carcinoma and 20% –40% of all breast cancers, as well as a substantial fraction of adenocarcinomas arising in the ovaries, prostate, colon and lung. HER-2/neu is intimately associated not only with the malignant phenotype, but also with the aggressiveness of the malignancy, being found in one-fourth of all invasive breast cancers. HER-2/neu overexpression is correlated with a poor prognosis in both breast and ovarian cancer. HER-2/neu is a transmembrane protein with a relative molecular mass of 185 kd that is approximately 1255 amino acids (aa) in length. It has an extracellular binding domain (ECD) of approximately 645 aa, with 40% homology to epidermal growth factor receptor (EGFR), a highly hydrophobic transmembrane anchor domain (TMD), and a carboxyterminal cytoplasmic domain (CD of approximately 580 aa with 80% homology to EGFR.

An approach to developing a diagnostic assay for malignancies, in which the HER-2/neu oncogene is associated, has been to attempt to quantify the protein expression product of the HER-2/neu oncogene in tissue or body fluids. However, there have been problems in the development of diagnostic assays based on direct detection of HER-2/neu protein.

Due to the difficulties in the current approaches to diagnosis and therapy of cancers in which the HER-2/neu oncogene is associated, there is a need in the art for improved methods and compositions. The present invention fills this need, and further provides other related advantages.

SUMMARY OF THE INVENTION

Briefly stated, the present invention provides a variety of methods for the detection of a malignancy in a warm-blooded animal, wherein a HER-2/neu oncogene is associated with the malignancy. The methods may be used on a one time basis when a malignancy is suspected or on a periodic basis, e.g., to monitor an individual with an elevated risk of acquiring or reacquiring a malignancy. In one embodiment, the method comprises the steps of: (a) isolating $CD4^+$ T cells from a warm-blooded animal; (b) incubating the T cells with HER-2/neu protein; and (c) detecting the presence or absence of specific activation of the T cells, thereby determining the presence or absence of the malignancy. In another embodiment, the method comprises the steps of: (a) isolating $CD8^+$ T cells from a warm-blooded animal; (b) incubating the T cells with HER-2/neu protein; and (c) detecting the presence or absence of specific activation of the T cells, thereby determining the presence or absence of the malignancy. In another embodiment, the method comprises the steps of: (a) contacting a body fluid, suspected of containing antibodies specific for HER-2/neu protein, with HER-2/neu protein; (b) incubating the body fluid under conditions and for a time sufficient to allow immunocomplexes to form; and (c) detecting the presence or absence of immunocomplexes formed between the HER-2/neu protein and antibodies in the body fluid specific for the HER-2/neu protein, thereby determining the presence or absence of the malignancy.

In another aspect, the present invention provides methods for monitoring the effectiveness of cancer therapy in a warm-blooded animal with a malignancy, wherein a HER-2/neu oncogene is associated with the malignancy. Uses of such methods include the early detection of relapse. In one embodiment, the method comprises the steps of: (a) contacting a first body fluid sample, taken from the warm-blooded animal prior to initiation of therapy, with HER-2/

neu protein; (b) incubating the body fluid under conditions and for a time sufficient to allow immunocomplexes to form; (c) detecting immunocomplexes formed between the HER-2/neu protein and antibodies in the body fluid specific for the HER-2/neu protein; (d) repeating steps (a), (b), and (c) on a second body fluid sample taken from the animal subsequent to the initiation of therapy; and (e) comparing the number of immunocomplexes detected in the first and second body fluid samples, thereby monitoring the effectiveness of the therapy in the animal.

The present invention is also directed toward methods for treating a malignancy in a warm-blooded animal, wherein a HER-2/neu oncogene is associated with the malignancy. In one embodiment, the method comprises the steps of: (a) isolating $CD4^+$ T cells from a warm-blooded animal; (b) incubating the T cells in the presence of HER-2/neu protein, such that the T cells proliferate; and (c) administering to the warm-blooded effective amount of the proliferated T cells. In another embodiment, the method comprises the steps of: (a) isolating $CD8^+$ T cells from a warm-blooded animal; (b) incubating the T cells in the presence of HER-2/neu protein, such that the T cells proliferate; and (c) administering to the warm-blooded animal an effective amount of the proliferated T cells. In another embodiment, the method comprises the steps of: (a) isolating $CD4^+$ T cells from a warm-blooded animal; (b) incubating the T cells in the presence of HER-2/neu protein, such that the T cells proliferate; (c) cloning one or more cells that proliferated in the presence of HER-2/neu protein; and (d) administering to the warm-blooded animal an effective amount of the cloned T cells. In another embodiment, the method comprises the steps of: (a) isolating $CD8^+$ T cells from a warm-blooded animal; (b) incubating the T cells in the presence of HER-2/neu protein, such that the T cells proliferate; (c) cloning one or more cells that proliferated in the presence of HmR-2/neu protein; and (d) administering to the warm-blooded animal an effective amount of the cloned T cells. In yet another embodiment, the method comprises immunizing the animal with a HER-2/neu peptide recognized by T cells, the peptide not being the extracellular domain of the protein expression product of a HER-2/neu oncogene.

Within a related aspect, the present invention provides anti-cancer therapeutic compositions comprising T cells proliferated in the presence of HER-2/neu protein, in combination with a pharmaceutically acceptable carrier or diluent. In addition, a variety of peptides designated for $CD8^+$ T cell responses are provided which include peptides consisting essentially of:

His-Leu-Tyr-Gln-Gly-Cys-Gln-Val-Val (Seq. ID No. 1);

Pro-Leu-Gln-Pro-Glu-Gln-Leu-Gln-Val (Seq. ID No. 2);

Pro-Leu-Thr-Ser-Ile-Ile-Ser-Ala-Val (Seq. ID No. 3);

Ile-Leu-Leu-Val-Val-Val-Leu-Gly-Val (Seq. ID No. 4);

Leu-Leu-Val-Val-Val-Leu-Gly-Vat-Val (Seq. ID No. 5);

Arg-Leu-Leu-Gln-Glu-Thr-Glu-Leu-Val (Seq. ID No. 6);

Cys-Leu-Thr-Ser-Thr-Val-Gln-Leu-Val (Seq. ID No. 7);

Asp-Leu-Ala-Ala-Arg-Asn-Val-Leu-Val (Seq. ID No. 8);

Val-Leu-Val-Lys-Ser-Pro-Asn-His-Val (Seq. ID No. 9);

Thr-Leu-Ser-Pro-Gly-Lys-Asn-Gly-Val (Seq. ID No. 10);

Val-Leu-Gly-Val-Val-Phe-Gly-Ile-Leu (Seq. ID No. 11);

Leu-Ile-Lys-Arg-Arg-Gln-Gln-Lys-Ile (Seq. ID No. 12);

Lys-Ile-Pro-Val-Ala-Ile-Lys-Val-Leu (Seq. ID No. 13);

Ile-Leu-Asp-Glu-Ala-Tyr-Val-Met-Ala (Seq. ID No. 14);

Gln-Leu-Met-Pro-Tyr-Gly-Cys-Leu-Leu (Seq. ID No. 15);

Gln-Ile-Ala-Lys-Gly-Met-Ser-Tyr-Leu (Seq. ID No. 16);

Leu-Leu-Asn-Trp-Cys-Met-Gln-Ile-Ala (Seq. ID No. 17);

Arg-Leu-Val-His-Arg-Asp-Leu-Ala-Ala (Seq. ID No. 18);

Asp-Ile-Asp-Glu-Thr-Glu-Tyr-His-Ala (Seq. ID No. 19);

Asp-Leu-Leu-Glu-Lys-Gly-Glu-Arg-Leu (Seq. ID No. 20);

Thr-Ile-Asp-Val-Tyr-Met-Leu-Met-Val (Seq. ID No. 21);

Met-Ile-Met-Val-Lys-Cys-Trp-Met-Ile (Seq. ID No. 22);

Asp-Leu-Val-Asp-Ala-Glu-Glu-Tyr-Leu (Seq. ID No. 23);

Gly-Leu-Glu-Pro-Ser-Glu-Glu-Glu-Ala (Seq. ID No. 24); or

Tyr-Leu-Thr-Pro-Gln-Gly-Gly-Ala-Ala (Seq. ID No. 25).

Similarly, a variety of peptides designated for $CD4^+$ T cell responses are provided which include peptides consisting essentially of:

His-Leu-Asp-Met-Leu-Arg-His-Leu-Tyr-Gln-Gly-Cys-Gln-Val-Val (Seq. ID No. 30);

Pro-Leu-Gln-Arg-Leu-Arg-Ile-Val-Arg-Gly-Thr-Gln-Leu-Phe-Glu (Seq. ID No. 31);

Leu-Arg-Ser-Leu-Thr-Glu-Ile-Leu-Lys-Gly-Gly-Val-Leu-Ile-Gln (Seq. ID No. 32);

Val-Thr-Tyr-Asn-Thr-Asp-Thr-Phe-Glu-Ser-Met-Pro-Asn-Pro-Glu (Seq. ID No. 33);

His-Leu-Arg-Glu-Val-Arg-Ala-Val-Thr-Ser-Ala-Asn-Ile-Gln-Glu (Seq. ID No. 34);

Val-Arg-Ala-Val-Thr-Ser-Ala-Asn-Ile-Gln-Glu-Phe-Ala-GlY-CYs (Seq. ID No. 35);

Asn-Ile-Gln-Glu-Phe-Ala-Gly-Cys-Lys-Lys-Ile-Phe-Gly-Ser-Leu (Seq. ID No. 36);

Gln-Val-Phe-Glu-Thr-Leu-Glu-Glu-Ile-Thr-Gly-Tyr-Leu-Tyr-Ile (Seq. ID No. 37);

Gln-Glu-Cys-Val-Glu-Glu-Cys-Arg-Val-Leu-Gln-Gly-Leu-Pro-Arg (Seq. ID No. 38);

Val-Val-Val-Leu-Gly-Val-Val-Phe-Gly-Ile-Leu-Ile-Lys-Arg-Arg (Seq. ID No. 39);

Lys-Tyr-Thr-Met-Arg-Arg-Leu-Leu-Gln-Glu-Thr-Glu-Leu-Val-Glu (Seq. ID No. 40);

Gly-Ala-Met-Pro-Asn-Gln-Ala-Gln-Met-Arg-Ile-Leu-Lys-Glu-Thr (Seq. ID No. 41);

Val-Lys-Val-Leu-Gly-Ser-Gly-Ala-Phe-Gly-Thr-Val-Tyr-Lys-Gly (Seq. ID No. 42);

Ser-pro-Lys-Ala-Asn-Lys-Glu-Ile-Leu-Asp-Glu-Ala-Tyr-Val-Met (Seq. ID No. 43);

Gly-Val-Gly-Ser-Pro-Tyr-Val-Ser-Arg-Leu-Leu-Gly-Ile-Cys-Leu (Seq. ID No. 44);

Ser-Arg-Leu-Leu-Gly-Ile-Cys-Leu-Thr-Ser-Thr-Val-Gln-Leu-Val (Seq. ID No. 45);

Gly-Ser-Gln-Asp-Leu-Leu-Asn-Trp-Cys-Met-Gln-Ile-Ala-Lys-Gly (Seq. ID No. 46);

Val-Lys-Ile-Thr-Asp-Phe-Gly-Leu-Ala-Arg-Leu-Leu-Asp-Ile-Asp (Seq. ID No. 47);

Thr-Val-Trp-Glu-Leu-Met-Thr-Phe-Gly-Ala-Lys-Pro-Tyr-Asp-Gly (Seq. ID No. 48);

Pro-Ala-Arg-Glu-Ile-Pro-Asp-Leu-Leu-Glu-Lys-Gly-Glu-Arg-Leu (Seq. ID No 49);

Arg-Phe-Arg-Glu-Leu-Val-Ser-Glu-Phe-Ser-Arg-Met-Ala-Arg-Asp (Seq. ID No 50);

Glu-Asp-Asp-Asp-Met-Gly-Asp-Leu-Val-Asp-Ala-Glu-Glu-Tyr-Leu (Seq. ID No 51);

Gly-Met-Gly-Ala-Ala-Lys-Gly-Leu-Gln-Ser-Leu-Pro-Thr-His-Asp (Seq. ID No 52);

Thr-Cys-Ser-Pro-Gln-Pro-Glu-Tyr-Val-Asn-Gln-Pro-Asp-Val-Arg (Seq. ID No 53);

Thr-Leu-Glu-Arg-Pro-Lys-Thr-Leu-Ser-Pro-Gly-Lys-Asn-Gly-Val Seq. ID No 54);

Gly-Gly-Ala-Val-Glu-Asn-Pro-Glu-Tyr-Leu-Thr-Pro-Gln-Gly-Gly Seq. ID No 55);

Asn-Gln-Glu-Val-Thr-Ala-Glu-Asp-Gly-Thr-Gln-Arg-Cys-Glu-Lys Seq. ID No 56);

Gln-Val-Ile-Arg-Gly-Arg-Ile-Leu-His-Asn-Gly-Ala-Tyr-Ser-Leu Seq. ID No 57);

Leu-Gln-Val-Phe-Glu-Thr-Leu-Glu-Glu-Ile-Thr-Gly-Tyr-Leu-Tyr Seq. ID No 58);

Ala-Ser-Pro-Leu-Thr-Ser-Ile-Ile-Ser-Ala-Val-Val-Gly-Ile-Leu Seq. ID No 59);

Thr-Gln-Arg-Cys-Glu-Lys-Cys-Ser-Lys-Pro-Cys-Ala-Arg-Val-Cys-Tyr-Gly-Leu (Seq. ID No. 60);

Arg-Leu-Arg-Ile-Val-Arg-Gly-Thr-Gln-Leu-Phe-Glu-Asp-Asn-Tyr-Ala-Leu (Seq. ID No. 61);

Lys-Ile-Phe-Gly-Ser-Leu-Ala-Phe-Leu-Pro-Glu-Ser-Phe-Asp-Gly-Asp (Seq. ID No. 62);

Arg-Arg-Leu-Leu-Gln-Glu-Thr-Glu-Leu-Val-Glu-Pro-Leu-Thr-Pro-Ser (Seq. ID No. 63); or

Glu-Leu-Val-Ser-Glu-Phe-Ser-Arg-Met-Ala-Arg-Asp-Pro-Gln (Seq. ID No. 64).

Additional peptides are provided and include a peptide consisting essentially of the amino acid sequence of FIG. 1 from lysine, amino acid 676, to valine, amino acid 1255.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows that $p185^{HER\text{-}2/neu}$ protein contains multiple segments with amino acid sequences appropriate for binding to class II MHC molecules. Each outlined amino acid represents the center point of an 11-mar peptide with alpha-helical periodicity and amphipathicity. Each underlined amino acid segment represents an epitope corresponding to Rothbard and Taylor motifs.

FIG. 2 graphically illustrates the results of a low frequency event screening from a normal individual indicating that a $CD4^+$ T cell response can be detected against $p185^{HER\text{-}2/neu}$ and peptides derived from its amino acid sequence. The graph represents the data from one normal individual analyzed with the low frequency screening assayed described further below. Positive responses to the intact protein and two peptides were detected.

FIGS. 3A–3D graphically illustrate that $CD4^+$ T cells reactive to $p185^{HER\text{-}2/neu}$ protein and peptides can be detected in high frequency from patients with HER-2/neu positive breast cancer and can also be detected in some patients with tumors that test negatively for expression of $p185^{HER\text{-}2/neu}$ protein. All four breast cancer patients represented here, patient A (FIG. 3A), patient B (FIG. 3B), patient C (FIG. 3C), and patient D (FIG. 3D), were premenopausal women. Patient A had a primary tumor that tested negatively for overexpression of $p185^{HER\text{-}2/neu}$. The other three patients had HER-2/neu positive tumors. A proliferation assay was performed using purified peripheral blood mononuclear cells (PBMC) as described below, with each experimental group done in 24 well replicate. Two$\times 10^5$ PBMC/well were incubated with no antigen, tetanus toxoid (5 μg/ml), $p185^{HER\text{-}2/neu}$ (5 μg/ml), or HER-2/neu derived peptides (50 μg/ml) as described further below. After 4 days, wells were pulsed with 1 μ Ci of tritiated thymidine (^{3}H-TdR) for 6–8 hours and then counted. The data represents the mean of 24 determinations of the c.p.m. with standard error bars expressed.

FIGS. 4A–4B graphically illustrate that $CD8^+$ CTL specific for HER-2/neu peptides 48–56 and 789–797 can be generated by in vitro immunization. Three$\times 10^7$ PBMC from a homozygous HLA-A2 normal donor were incubated with p48–56 or p789–797HER-2/neu peptides at concentrations of 10 μg/ml. The lymphocytes were tested for lytic activity after 10 in vitro sensitizations (IVS). Data is depicted after the tenth IVS with p48–56 (FIG. 4A) or with p789–797 (FIG. 4B). Target cells consisted of ^{51}Cr-labeled autologous EBV transformed B lymphocytes which had been incubated with p48–56 or p789–797^{HER}-2/neu or an irrelevant peptide for 2 hours prior to use. A four hour chromium release assay (CRA) was performed. The results represent the percent specific lysis at the indicated effector:target (E:T) ratio. Target controls of ^{51}Cr-labeled K562 and Daudi cells were also included to evaluate NK and LAK activity. The execution of the CRA is as described. The results represent the percent specific lysis at the indicated effector:target (E:T) ratio.

FIG. 5 pictorially illustrates that antibodies are detectable against $p185^{HER\text{-}2/neu}$ in the sera of a breast cancer patient. Lane 1 represents the immunoblot of p185 using a HER-2/neu positive breast cancer patient's sera (1:1000 dilution) as primary antibody. The blot was analyzed as described further below. Lane 2 represents the control strip from that experiment developed with c-neu antibody.

FIG. 6 pictorially shows that antibodies in the sera of a breast cancer patient identify the same p185 band as does a known HER-2/neu-specific antibody ("control antibody"). A membrane preparation from NIH3T3 cells (a murine cell line) that had been transfected with HER-2/neu cDNA ("NIH3T3+H2N") was tested against control antibody (lane A) or patient sera (lane D). Similarly, a membrane preparation from untransfected cells ("NIH3T3") was tested against control antibody (lane B) or patient sera (lane C).

FIG. 7 pictorially illustrates that some breast cancer patients have antibodies directed to both the extracellular and intracellular domain of the HER-2/neu protein. Sera of breast cancer patients is tested against the extracellular domain ("ECD protein") or the intracellular domain ("ICD protein"), in lanes A and B, respectively.

FIG. 8 graphically illustrates that rats immunized with peptides derived from the intracellular domain (ICD) portion of rat neu protein develop antibody responses to neu protein. An ELISA was performed to evaluate peptide immunized animals for antibody responses to non-transforming rat neu protein. Each sera was analyzed at a 1:25, 1:50, 1:100, and 1:200 dilution. The OD value shown is that of the background wells subtracted from the wells coated with neu protein. All data shown is at a rat sera concentration of 1:25. Control sera was derived from an animal immunized with adjuvant alone. Antibody responses titered with decreasing serum concentrations. Results were reproducible in 3 separately run assays.

FIG. 9 graphically illustrates that rats immunized with peptides derived from the extracellular domain (ECD) portion of rat neu protein develop antibody responses to neu protein. ELISA evaluation was performed as described in FIG. 8. All data shown is at a rat sera concentration of 1:25. Control sera was derived from an animal immunized with

adjuvant alone. Antibody responses titered with decreasing serum concentrations. Results were reproducible in 3 separately run assays.

FIG. **10** graphically shows that epitope analysis of ICD antibody responses demonstrates dominant B cell epitopes as well as "determinant spreading" between domains. ELISA analysis for peptide epitopes was performed. Each animal's sera was evaluated at dilutions of 1:25, 1:50, 1:100, and 1:200 for each peptide analyzed. Antibody responses titered with decreasing serum concentrations. All data shown is at a rat sera concentration of 1:50. Control sera analyzed was pooled sera from 5 non-immunized animals. Results were reproducible in 3 separately run assays.

FIG. **11** graphically shows that epitope analysis of ECD antibody responses demonstrates dominant B cell epitopes. ELISA analysis for peptide epitopes was performed. Each animal's sera was evaluated at dilutions of 1:25, 1:50, 1:100, and 1:200 for each peptide analyzed. Antibody responses titered with decreasing serum concentrations. All data shown is at a rat sera concentration of 1:50. Control sera analyzed was pooled sera from 5 non-immunized animals. Results were reproducible in 3 separately run assays.

FIG. **12** pictorially illustrates that antibodies elicited by immunization to either ICD or ECD peptides are specific for and can immunoprecipitate both rat neu protein and human HER-2/neu protein. FIG. **12A** shows the results of an immunoprecipitation experiment with immunized rat sera and lysates of DHFRG-8. Each sera was able to immunoprecipitate rat neu from the cell lysates. The immunoprecipitates were resolved on a 7.5% SDS-acrylamide gel and transferred to nitrocellulose. The blots were probed with primary antibody, c-neu-Ab-3, at a 1:1000 dilution. Control sera of an animal immunized with the adjuvant alone showed no evidence of reactivity to rat neu. FIG. **12B** depicts the results of an immunoprecipitation experiment with immunized rat sera and lysates of SKBR3, a source of human neu. Immunoblotting was performed in an identical manner and all experimental animal sera were able to immunoprecipitate human neu. The control sera, again, showed no evidence of reactivity.

FIG. **13** pictorially illustrates that B cell epitopes that are cross reactive between human and rat neu are present in both domains of the protein. Shown here are the results of Western blot analysis of protein domain epitope mapping from representative animals in each immunized group. Animal 1.2 was immunized with the ECD group of peptides, animal 2.2 with the ICD group of peptides, and the control animal was immunized with adjuvant alone. Proteins were electrophoresed. After transfer to nitrocellulose the blots were incubated for 18 hours in rat sera at a 1:500 dilution. Antibody responses were detected with a second step goat anti-rat Ig HRP antibody at a dilution of 1:5000. Responses were detected to both human and rat neu as well as to both human ICD and ECD domain recombinant proteins. Antibody responses to these proteins could not be detected in the control animal which was immunized with adjuvant alone. Although data are shown here for animals 1.2, 2.2, and control, all animals in each group had the same pattern of response.

FIG. **14** graphically illustrates that immunization of rats with ICD peptides elicits neu peptide-specific T cell responses. 2×10^5 immunized spleen cells were incubated with 25 μg/ml of the various peptides. The "Mix" group consisted of 25 μg/ml each of the immunizing peptides. A proliferation assay was performed. Each experimental group was done in 6 well replicates. The data is expressed in terms of a stimulation index (SI) which is the mean of the experimental wells divided by the mean of the control (no antigen) wells. Stimulation indices greater than 2 are considered to be indicative of a primed response. Animals immunized with adjuvant alone showed no stimulation index greater than 0.9 to any of the tested peptides (data not shown).

FIG. **15** graphically illustrates that immunization of rats with ICD peptides elicits neu protein-specific T cell responses. 1×10^5 cultured T cells derived from immunized spleen were incubated with 1×10^5 syngeneic spleen as APC (antigen presenting cells) and 1 μg/ml of purified rat neu protein. Each experimental group was done in 6 well replicates. The data is expressed in terms of a stimulation index which is the mean of the experimental wells divided by the mean of the control (no antigen) wells. Stimulation indices greater than 2 are considered to be indicative of a primed response. Wild type ras protein was the irrelevant protein used in the assay.

FIG. **16** graphically shows that immunization of rats with ECD peptides elicits only weak peptide-specific T cell responses. 2×10^5 immunized spleen cells were incubated with 25 μg/ml of the various peptides. The "Mix" group consisted of 25 μg/ml each of the immunizing peptides. Each experimental group was done in 6 well replicates. The data is expressed in terms of a stimulation index which is the mean of the experimental wells divided by the mean of the control (no antigen) wells. Stimulation indices greater than 2 are considered to be indicative of a primed response. Animals immunized with adjuvant alone showed no stimulation index greater than 1.0 to any of the tested peptides (data not shown).

FIG. **17** graphically shows that immunization of rats with ECD peptides elicits weak, but positive, responses to neu protein. 1×10^5 cultured T cells derived from immunized spleen or lymph nodes were incubated with 1×10^5 syngeneic spleen as APC and 1 μg/ml of purified rat neu protein. Each experimental group was done in 6 well replicates. The data is expressed in terms of a stimulation index which is the mean of the experimental wells divided by the mean of the control (no antigen) wells. Stimulation indices greater than 2 are considered to be indicative of a primed response. Wild type ras protein was the irrelevant protein used in the assay.

DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

HER-2/neu Protein—as used herein, refers to the p185 protein (also known as c-erbB2) and peptides thereof which are recognized by helper T cells or cytotoxic T cells; and may be naturally derived, synthetically produced, genetically engineered, or a functional equivalent thereof, e.g., where one or more amino acids are replaced by other amino acid(s) or non-amino acid(s) which do not substantially affect function.

Proliferation of T cells—as used herein, includes the multiplication of T cells as well as the stimulation of T cells leading to multiplication, i.e., the initiation of events leading to mitosis and mitosis itself. Methods for detecting proliferation of T cells are discussed below.

As noted above, the present invention is directed toward methods and compositions for the diagnosis, monitoring and treatment of malignancies in a warm-blooded animal, wherein an amplified HER-2/neu gene is associated with the

malignancies. Association of an amplified HER-2/neu gene with a malignancy does not require that the protein expression product of the gene be present on the tumor. For example, overexpression of the protein expression product may be involved with initiation of a tumor, but the protein expression may subsequently be lost. An effective autochthonous immune response may convert a HER-2/neu positive tumor to HER-2/neu negative, but existent immunity will be present and allow diagnosis.

More specifically, the disclosure of the present invention, in one aspect, shows that the protein expression product of the HER-2/neu gene can be recognized by thymus-dependent lymphocytes (hereinafter "T cells") and, therefore, the autochthonous immune T cell response can be utilized to diagnose, monitor and treat malignancies in which such a protein is or has been overexpressed. The disclosure of the present invention also shows, in another aspect, that sera of patients with a malignancy, in which an amplified HER-2/neu oncogene is associated, contain antibodies to HER-2/neu protein. The autochthonous antibody response can be used to diagnose, monitor and treat malignancies in which such a protein is overexpressed.

It is well known that the two major arms of the immune system are: (1) cell-mediated immunity with immune T cells and (2) humoral immunity with antibodies. Further, the immune system normally functions to recognize and destroy any foreign or aberrant cells in the body. Since the HER-2/neu protein is expressed by some normal cells, tolerance and/or anergy (i.e., diminished reactivity to a specific antigen) is expected. Thus, it is surprising that, as disclosed within the present invention, both T cell and antibody responses to HER-2/neu are detected.

In general, $CD4^+$ T cell populations are considered to function as helpers/inducers through the release of lymphokines when stimulated by a specific antigen; however, a subset of $CD4^+$ cells can act as cytotoxic T lymphocytes (CTL). Similarly, $CD8^+$ T cells are considered to function by directly lysing antigenic targets; however, under a variety of circumstances they can secrete lymphokines to provide helper or DTH function. Despite the potential of overlapping function, the phenotypic CD4 and CD8 markers are linked to the recognition of peptides bound to class II or class I MHC antigens. The recognition of antigen in the context of class II or class I MHC mandates that $CD4^+$ and $CD8^+$ T cells respond to different antigens or the same antigen presented under different circumstances. The binding of immunogenic peptides to class II MHC antigens most commonly occurs for antigens ingested by antigen

Therefore, $CD4^+$ T cells generally presenting cells. Therefor, CD^+ T cells generally recognize antigens that have been external to the tumor cells. By contrast, under normal circumstances, binding of peptides to class I MHC occurs only for proteins present in the cytosol and synthesized by the target itself, proteins in the external environment are excluded. An exception to this is the binding of exogenous peptides with a precise class I binding motif which are present outside the cell in high concentration. Thus, $CD4^+$ and $CD8^+$ T cells have broadly different functions and tend to recognize different antigens as a reflection of where the antigens normally reside.

As disclosed within the present invention, the protein product expressed by the HER-2/neu oncogene is recognized by T cells. Such a protein expression product "turns over" within cells, i.e., undergoes a cycle wherein a synthesized protein functions and then eventually is degraded and replaced by a newly synthesized molecule. During the protein life cycle, peptide fragments from the protein bind to major histocompatibility complex (MHC) antigens. By display of a peptide bound to MHC antigen on the cell surface and recognition by host T cells of the combination of peptide plus self MHC antigen, a malignant cell will be immunogenic to T cells. The exquisite specificity of the T cell receptor enables individual T cells to discriminate between protein fragments which differ by a single amino acid residue.

During the immune response to a peptide, T cells expressing a T cell receptor with high affinity binding of the peptide-MHC complex will bind to the peptide-MHC complex and thereby become activated and induced to proliferate. In the first encounter with a peptide, small numbers of immune T cells will secrete lymphokines, proliferate and differentiate into effector and memory T cells. The primary immune response will occur in vivo but has been difficult to detect in vitro. Subsequent encounter with the same antigen by the memory T cell will lead to a faster and more intense immune response. The secondary response will occur either in vivo or in vitro. The in vitro response is easily gauged by measuring the degree of proliferation, the degree of cytokine production, or the generation of cytolytic activity of the T cell population re-exposed in the antigen. Substantial proliferation of the T cell population in response to a particular antigen is considered to be indicative of prior exposure or priming to the antigen.

Within one aspect of the present invention, a malignancy in which a HER-2/neu oncogene is associated may be detected. Representative examples of such malignancies include breast, ovarian, colon, lung and prostate cancers. An immune response to the HER-2/neu protein, once generated, can be long-lived and can be detected long after immunization, regardless of whether the protein is present or absent in the body at the time of testing. In one embodiment, prior exposure of a warm-blooded animal, such as humans, to the HER-2/neu protein can be detected by examining for the presence or absence of specific activation of $CD4^+$ or $CD8^+$ T cells. More specifically, T cells isolated from an individual by routine techniques (such as by Ficoll/Hypaque density gradient centrifugation of peripheral blood lymphocytes) are incubated with HER-2/neu protein. For example, T cells may be incubated in vitro for 2–9 days (typically 4 days) at 37° C. with HER-2/neu protein (typically, 5 μg/ml of whole protein or 25 μg/ml of an appropriate peptide or graded numbers of cells synthesizing HER-2/neu protein). It may be desirable to incubate another aliquot of a T cell sample in the absence of HER-2/neu protein to serve as a control.

Specific activation of $CD4^+$ or $CD8^+$ T cells may be detected in a variety of ways. Methods for detecting specific T cell activation include detecting the proliferation of T cells, the production of cytokines (e.g., lymphokines), or the generation of cytolytic activity (i.e., generation of cytotoxic T cells specific for HER-2/neu protein). For $CD4^+$ T cells, a preferred method for detecting specific T cell activation is the detection of the proliferation of T cells. For $CD8^+$ T cells, a preferred method for detecting specific T cell activation is the detection of the generation of cytolytic activity.

Detection of the proliferation of T cells may be accomplished by a variety of known techniques. For example, T cell proliferation can be detected by measuring the rate of DNA synthesis. T cells which have been stimulated to proliferate exhibit an increased rate of DNA synthesis. A typical way to measure the rate of DNA synthesis is, for example, by pulse-labeling cultures of T cells with tritiated thymidine, a nucleoside precursor which is incorporated into

newly synthesized DNA. The amount of tritiated thymidine incorporated can be determined using a liquid scintillation spectrophotometer. Other ways to detect T cell proliferation include measuring increases in interleukin-2 (IL-2) production, Ca^{2+} flux, or dye uptake, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium. Alternatively, synthesis of lymphokines (such as interferon-gamma) can be measured or the relative number of T cells that can respond to intact $p185^{HER\text{-}2/neu}$ protein or peptide may be quantified.

Intact $p185^{HER\text{-}2/neu}$ protein or peptides thereof which are recognized by cytotoxic T cells may be used within the present invention. The peptides may be naturally derived or produced based upon an identified sequence. The peptides for $CD8^+$ T cell responses are generally 8–10 amino acids in length and the peptides for $CD4^+$ T cell responses are longer, e.g., 15–18 amino acids in length. Peptides for $CD8^+$ T cell responses vary according to each individual's class I MHC molecules. An example of peptides appropriate for $CD8^+$ T cell responses (elicited by peptides presented by HLA-A2.1 class I MHC molecules) are peptides which are 8–10 amino acids in length and contain a leucine at position **2** and/or a leucine or valine at position **9**. Examples of peptides (designated by one letter abbreviations for amino acids and followed in parentheses by which residues of p185 they correspond) suitable within the present invention for $CD8^+$ T cell responses in individuals that are HLA-A2.1 include peptides consisting essentially of: HLYQGCQVV (p48–56) (Seq. ID No. 1); QLFEDNYAL (p106–114) (Seq. ID No. 26); KIFGSLAFL (p369–377) (Seq. ID No. 27); PLQPEQLQV (p391–399) (Seq. ID No. 2); PLTSIISAV (p650–658) (Seq. ID No. 3); ILLVVVLGV (p66–669) (Seq. ID No. 4); LLVVVLGVV (p662–670) (Seq. ID No. 5); RLLQETELV (p689–697) (Seq. ID No. 6); ILDEAYVMAGV (p767–777) (Seq. ID No. 28); VMAGVGSPYV (p773–782) (Seq. ID No. 29); CLTSTVQLV (p789–797) (Seq. ID No. 7); DLAARNVLV (p845–853) (Seq. ID No. 8); VLVKSPNHV (p851–859) (Seq. ID No. 9); TLSPGKNGV (p1172–1180) (Seq. ID No. 10); VLGVVFGIL (p666–674) (Seq. ID No. 11); LIKRRQQKI (p674–682) (Seq. ID No. 12); KIPVAIKVL (p747–755) (Seq. ID No. 13); ILDEAYVMA (p767–775) (Seq. ID No. 14); QLMPYGCLL (p799–807) (Seq. ID No. 15); QIAKGMSYL (p829–836) (Seq. ID No. 16); LLNWCMQIA (p822–830) (Seq. ID No. 17); RLVHRDLAA (p840–848) (Seq. ID No. 18); DIDETEYHA (p871–879) (Seq. ID No. 19); DLLEKGERL (p933–941) (Seq. ID No. 20); TIDVYMLMV (p948–956) (Seq. ID No. 21); MIMVKCWMI (p953–961) (Seq. ID No. 22); DLVDAEEYL (p1016–1024) (Seq. ID No. 23); GLEPSEEEA (p1062–1070) (Seq. ID No. 24); or YLTPQGGAA (p1196–1204) (Seq. ID No. 25).

Peptides for $CD4^+$ T cell responses vary according to each individual's class II MHC molecules. Examples of peptides suitable within the present invention for $CD4^+$ T cell responses include peptides consisting essentially of: HLDMLRHLYQGCQVV (p42–56) (Seq. ID No. 30); PLQRLRIVRGTQLFE (p95–109) (Seq. ID No. 31); RLRIVRGTQLFEDNYAL (p98–114) (Seq. ID No. 61); LRSLTEILKGGVLIQ (p142–156) (Seq. ID No. 32); VTYNTDTFESMPNPE (p272–286) (Seq. ID No. 33); NQEVTAEDGTQRCEK (p319–333) (Seq. ID No. 56); TQRCEKCSKPCARVCYGL (p328–345) (Seq. ID No. 60); HLREVRAVTSANIQE (p349–363) (Seq. ID No. 34; VRAVTSANIQEFAGC (p353–367) (Seq. ID No. 35); NIQEFAGCKKIFGSL (p360–374) (Seq. ID No. 36); KIFGSLAFLPESFDGD (p369–384) (Seq. ID No. 62); LQVFETLEEITGYLY (p397–411) (Seq. ID No. 58); QVFETLEEITGYLYI (p398–412) (Seq. ID No. 37); QVIRGRILHNGAYSL (p429–443) (Seq. ID No. 57); QECVEECRVLQGLPR (p538–552) (Seq. ID No. 38); ASPLTSIISAVVGIL (p648–662) (Seq. ID No. 59); VVVLGVVFGILIKRR (p664–678) (Seq. ID No. 39); KYTMRRLLQETELVE (p684–698) (Seq. ID No. 40); RRLLQETELVEPLTPS (p688–703) (Seq. ID No. 63); GAMPNQAQMRILKET (p704–718) (Seq. ID No 41); VKVLGSGAFGTVYKG (p723–737) (Seq. ID No 42); SPKANKEILDEAYVM (p760–774) (Seq. ID No 43); GVGSPYVSRLLGICL (p776–790) (Seq. ID No 44); SRLLGICLTSTVQLV (p783–797) (Seq. ID No 45); GSQDLLNWCMQIAKG (p818–832) (Seq. ID No 46); VKITDFGLARLLDID (p859–873) (Seq. ID No 47); TVWELMTFGAKPYDG (p911–925) (Seq. ID No. 48); PAREIPDLLEKGERL (p927–941) (Seq. ID No. 49); RFRELVSEFSRMARD (p968–982) (Seq. ID No. 50); ELVSEFSRMARDPQ (p971–984) (Seq. ID No. 64); EDDDMGDLVDAEEYL (p1010–1024) (Seq. ID No. 51); GMGAAKGLQSLPTHD (p1091–1105) (Seq. ID No. 52); TCSPQPEYVNQPDVR (p1132–1146) (Seq. ID No. 53); TLERPKTLSPGKNGV (p1166–1180) (Seq. ID No. 54); or GGAVENPEYLTPQGG (p1188–1202) (Seq. ID No. 55).

It will be evident to those of ordinary skill in the art that other peptides may be produced for use within the present invention, both for the HLA-A2.1 class I MHC molecule as well as for the other class I and class II molecules. A variety of techniques are well known for isolating or constructing peptides. Suitable peptides are readily identified based upon the disclosure provided herein. Additional suitable peptides include those which are longer in length. For example, another peptide has an amino acid sequence corresponding to that disclosed in FIG. **1** beginning at about the lysine residue at amino acid position **676** and extending to about the valine residue at amino acid position **1255**. Such a peptide may be extended (e.g., by the addition of one or more amino acid residues selected, for example, from position **675** to about position **646** of FIG. **1**) and/or truncated (e.g., by the deletion of one or more amino acid residues from the carboxyl terminus which is position **1255** of FIG. **1**). Alternatively, suitable peptides may be variations on other preferred peptides disclosed herein. For example, variations on the peptide designated herein as p650–658 include the extension and/or truncation by the addition or deletion, respectively, of one or more amino acid residues beginning at either position **650** or position **658** or both positions. As an example, four amino acids are removed from the amino terminus of p650–658 and four amino acids, such as the four adjacent to position **658**, are added to its carboxylterminus. Although this particular peptide variation results in a peptide with the same number of total amino acids (nine), a peptide variation on a preferred peptide need not be identical in length. Variations in amino acid sequence that yield peptides having substantially the same desired biological activity are within the scope of the present invention.

For therapeutic purposes, $CD4^+$ or $CD8^+$ T cells that proliferate in the presence of HER-2/neu protein can be expanded in number either in vitro or in vivo. Proliferation of such T cells in vitro may be accomplished in a variety of ways. For example, the T cells can be re-exposed to HER-2/neu protein. It may be desirable to repeat the exposure of T cells to the HER-2/neu protein to induce proliferation. It may be further desirable to include T cell growth factors, such as interleukin-2, and/or stimulator cells which synthesize HER-2/neu protein. The addition of stimulator cells is

preferred where generating $CD8^+$ T cell responses. HER-2/neu protein-specific T cells can be grown to large numbers in vitro with retention of specificity in response to intermittent restimulation with the immunizing HER-2/neu protein. Briefly, for the primary in vitro stimulation (IVS), large numbers of lymphocytes (e.g., greater than 4×10^7) are placed in flasks with media containing human serum. HER-2/neu protein (e.g., peptide at 10 μg/ml) is added directly as well as 5 μg/ml tetanus toxoid. The flasks are incubated at 37° C. for 7 days. For the second IVS, at the end of the 7 days, T cells are harvested and placed in new flasks with 2–3 $\times10^7$ irradiated peripheral blood mononuclear cells. HER-2/neu protein (e.g., peptide at 10 μg/ml is added directly). The flasks are incubated at 37° C. for 7 days. On day 2 and day 4 after the second IVS, 2–5 units of interleukin-2 (IL-2) is added. For the third IVS, the T cells are placed in wells (e.g., 24 well plates). The T cells are stimulated with the individual's own EBV transformed B cells coated with the peptide. IL-2 is added on days 2 and 4 of each cycle. As soon as the cells are shown to be specific cytotoxic T cells, they are changed to a 10 day stimulation cycle with higher IL-2 (20 units) on days 2, 4 and 6 to expand them.

Alternatively, one or more T cells that proliferate in the presence of HER-2/neu protein can be expanded in number by cloning. Methods for cloning cells are well known in the art. For example, T cell lines may be established in vitro and cloned by limiting dilution. Responder T cells are purified from the peripheral blood of sensitized patients by density gradient centrifugation and sheep red cell rosetting and established in culture by stimulating with the nominal antigen in the presence of irradiated autologous filler cells. In order to generate $CD4^+$ T cell lines, HER-2/neu protein is used as the antigenic stimulus and autologous peripheral blood lymphocytes (PBL) or lymphoblastoid cell lines (LCL) immortalized by infection with Epstein Barr virus are used as antigen presenting cells. In order to generate $CD8^+$ T cell lines, autologous antigen-presenting cells transfected with an expression vector which produces relevant HER-2/neu protein may be used as stimulator cells. Established T cell lines are cloned 2–4 days following antigen stimulation by plating stimulated T cells at a frequency of 0.5 cells per well in 96-well flat-bottom plates with 1×10^6 irradiated PBL or LCL cells and recombinant interleukin-2 (rIL2) (50 U/ml). Wells with established clonal growth are identified at approximately 2–3 weeks after initial plating and restimulated with appropriate antigen in the presence of autologous antigen-presenting cells, then subsequently expanded by the addition of low doses of rIL2 (10 U/ml) 2–3 days following antigen stimulation. T cell clones are maintained in 24-well plates by periodic restimulation with antigen and rIL2 approximately every two weeks.

Regardless of how an individual's T cells are proliferated in vitro, the T cells may be administered to the individual as an anti-cancer composition in an amount effective for therapeutic attack against a tumor. Thus, a patient's own T cells (autochthonous T cells) can be used as reagents to mediate specific tumor therapy. Typically, about 1×10^9 to 1×10^{11} T cells/M^2 will be administered intravenously or intracavitary, e.g., in pleural or peritoneal cavities, or in the bed of a resected tumor. It will be evident to those skilled in the art that the number and frequency of administration will be dependent upon the response of the patient. Pharmaceutically suitable carriers or diluents for T cells include physiological saline or sera. It will be recognized by one skilled in the art that the composition should be prepared in sterile form.

T cells may also be proliferated in vivo. For example, immunization of an individual with a HER-2/neu peptide (i.e., as a vaccine) can induce continued expansion in the number of T cells necessary for therapeutic attack against a tumor in which the HER-2/neu oncogene is associated. Typically, about 0.01 μg/kg to about 100 mg/kg body weight will be administered by the intradermal, subcutaneous or intravenous route. A preferred dosage is about 1 μg/kg to about 1 mg/kg, with about 5 μg/kg to about 200 μg/kg particularly preferred. It will be evident to those skilled in the art that the number and frequency of administration will be dependent upon the response of the patient. It may be desirable to administer the HER-2/neu peptide repetitively. It will be evident to those skilled in this art that more than one HER-2/neu peptide may be administered, either simultaneously or sequentially. For example, a combination of about 8–15 peptides may be used for immunization. Preferred peptides for immunization are those that include all or a portion of the amino acid sequence shown in FIG. 1 beginning at about the lysine residue at amino acid position **676** and extending to about the valine residue at amino acid position **1255**. One or more peptides from other portions of the amino acid sequence shown in FIG. 1 may be added to one or more of the preferred peptides. Neither intact $p185^{HER\text{-}2/neu}$ protein nor a peptide having the amino acid sequence of its entire extracellular domain (i.e., a peptide having an amino acid sequence of the entire amino acid sequence shown in FIG. 1 up to amino acid position **650**, plus or minus about one to five positions, and with or without the first 21 amino acid positions) are used alone for immunization.

In addition to the HER-2/neu peptide (which functions as an antigen), it may be desirable to include other components in the vaccine, such as a vehicle for antigen delivery and immunostimulatory substances designed to enhance the protein's immunogenicity. Examples of vehicles for antigen delivery include aluminum salts, water-in-oil emulsions, biodegradable oil vehicles, oil-in-water emulsions, biodegradable microcapsules, and liposomes. Examples of immunostimulatory substances (adjuvants) include N-acetylmuramyl-L-alanine-D-isoglutamine (MDP), lipopoly-saccharides (LPS), glucan, IL-12, GM-CSF, gamma interferon and IL-15. It will be evident to those skilled in this art that a HER-2/neu peptide may be prepared synthetically or that a portion of the protein (naturally-derived or synthetic) may be used. When a peptide is used without additional sequences, it may be desirable to couple the peptide hapten to a carrier substance, such as keyhole limpet hemocyanin.

The present invention also discloses that HER-2/ neu protein, in addition to being immunogenic to T cells, appears to stimulate B-cells to produce antibodies capable of recognizing HER-2/neu protein. Detection of such antibodies provides another way to diagnose a malignancy in which a HER-2/neu oncogene is associated with the malignancy. Antibodies specific (i.e., which exhibit a binding affinity of about 10^7 liters/mole or better) for HER-2/neu protein may be found in a variety of body fluids including sera and ascites. Briefly, a body fluid sample is isolated from a warm-blooded animal, such as a human, for whom it is desired to determine whether antibodies specific for HER-2/neu are present. The body fluid is incubated with HER-2/neu protein under conditions and for a time sufficient to permit immunocomplexes to form between the protein and antibodies specific for the protein. For example, a body fluid and HER-2/neu protein may be incubated at 4° C. for 24–48 hours. Following the incubation, the reaction mixture is tested for the presence of immunocomplexes. Detection of one or more immunocomplexes formed between HER-2/neu

protein and antibodies specific for HER-2/neu protein may be accomplished by a variety of known techniques, such as radioimmunoassays (RIA) and enzyme linked immunosorbent assays (ELISA).

Suitable immunoassays include the double monoclonal antibody sandwich immunoassay technique of David et al. (U.S. Pat. No. 4,376,110); monoclonal-polyclonal antibody sandwich assays (Wide et al., in Kirkham and Hunter, eds., *Radioimmunoassay Methods*, E. and S. Livingstone, Edinburgh, 1970); the "western blot" method of Gordon et al. (U.S. Pat. No. 4,452,901); immunoprecipitation of labeled ligand (Brown et al., *J. Biol. Chem.* 255:4980–4983, 1980); enzyme-linked immunosorbent assays as described by, for example, Raines and Ross (J. Biol. Chem. 257:5154–5160, 1982); immunocytochemical techniques, including the use of fluorochromes (Brooks et al., *Clin. Exp. Immunol.* 39: 477, 1980); and neutralization of activity [Bowen-Pope et al., *Proc. Natl. Acad. Sci.* USA 81:2396–2400 (1984)], all of which are hereby incorporated by reference. In addition to the immunoassays described above, a number of other immunoassays are available, including those described in U.S. Pat. Nos.: 3,817,827; 3,850,752; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; and 4,098,876, all of which are herein incorporated by reference.

For detection purposes, HER-2/neu protein ("antigen") may either be labeled or unlabeled. When unlabeled, the antigen find use in agglutination assays. In addition, unlabeled antigen can be used in combination with labeled molecules that are reactive with immunocomplexes, or in combination with labeled antibodies (second antibodies) that are reactive with the antibody directed against HER-2/neu protein, such as antibodies specific for immunoglobulin. Alternatively, the antigen can be directly labeled. Where it is labeled, the reporter group can include radioisotopes, fluorophores, enzymes, luminescers, or dye particles. These and other labels are well known in the art and are described, for example, in the following U.S. Pat. Nos.: 3,766,162; 3,791,932; 3,817,837; 3,996,345; and 4,233,402.

Typically in an ELISA assay, antigen is adsorbed to the surface of a microtiter well. Residual protein-binding sites on the surface are then blocked with an appropriate agent, such as bovine serum albumin (BSA), heat-inactivated normal goat serum (NGS), or BLOTTO (buffered solution of nonfat dry milk which also contains a preservative, salts, and an antifoaming agent). The well is then incubated with a sample suspected of containing specific antibody. The sample can be applied neat, or, more often, it can be diluted, usually in a buffered solution which contains a small amount (0.1% –5.0% by weight) of protein, such as BSA, NGS, or BLOTTO. After incubating for a sufficient length of time to allow specific binding to occur, the well is washed to remove unbound protein and then incubated with an anti-species specific immunoglobulin antibody labeled with a reporter group. The reporter group can be chosen from a variety of enzymes, including horseradish peroxidase, beta-galactosidase, alkaline phosphatase, and glucose oxidase. Sufficient time is allowed for specific binding to occur, then the well is again washed to remove unbound conjugate, and the substrate for the enzyme is added. Color is allowed to develop and the optical density of the contents of the well is determined visually or instrumentally.

In one preferred embodiment of this aspect of the present invention, a reporter group is bound to HER-2/neu protein. The step of detecting immunocomplexes involves removing substantially any unbound HER-2/neu protein and then detecting the presence or absence of the reporter group.

In another preferred embodiment, a reporter group is bound to a second antibody capable of binding to the antibodies specific for HER-2/neu protein. The step of detecting immunocomplexes involves (a) removing substantially any unbound antibody, (b) adding the second antibody, (c) removing substantially any unbound second antibody and then (d) detecting the presence or absence of the reporter group. Where the antibody specific for HER-2/neu protein is derived from a human, the second antibody is an anti-human antibody.

In a third preferred embodiment for detecting immunocomplexes, a reporter group is bound to a molecule capable of binding to the immunocomplexes. The step of detecting involves (a) adding the molecule, (b) removing substantially any unbound molecule, and then (c) detecting the presence or absence of the reporter group. An example of a molecule capable of binding to the immunocomplexes is protein A.

It will be evident to one skilled in the art that a variety of methods for detecting the immunocomplexes may be employed within the present invention. Reporter groups suitable for use in any of the methods include radioisotopes, fluorophores, enzymes, luminescers, and dye particles.

In a related aspect of the present invention, detection of immunocomplexes formed between HER-2/neu protein and antibodies in body fluid which are specific for HER-2/neu protein may be used to monitor the effectiveness of cancer therapy for a malignancy in which the HER-2/neu oncogene is associated. Samples of body fluid taken from an individual prior to and subsequent to initiation of therapy may be analyzed for the immunocomplexes by the methodologies described above. Briefly, the number of immunocomplexes detected in both samples are compared. A substantial change in the number of immunocomplexes in the second sample (post-therapy initiation) relative to the first sample (pre-therapy) reflects successful therapy.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1

CD4+T Cells Responsive to p185(HER-2/neu) Protein and Peptides Can Be Detected in Higher Frequency in Patients with Breast Cancer than Normal Individuals

A. p185(HER-2/neu) Protein Contains Multiple Segments with Amino Acid Sequences Appropriate for Binding to Class II MHC Molecules

Soluble proteins are classically processed in the class II MHC pathway. $p185^{HER\text{-}2/neu}$ protein is a transmembrane protein present at the cell surface. When overexpressed, it has been found to be soluble and in the extracellular environment both in vitro and in vivo. In vitro studies of human breast cancer cell lines found the extracellular domain of $p185^{HER\text{-}2/neu}$ in culture media of rapidly growing cells (Alper et al., *Cell Growth and Differentiation* 1:591–599, 1990; Zabrecky et al., *J. Biol. Chem.* 266:1716–1720, 1991). In vivo studies identified circulating portions of the protein in the sera of patients with breast cancer (Leitzel et al., *J. Clin. Oncol.* 10:1436–1443, 1992; Mori et al., *Jpn. J. Cancer Res.* 81:489–494, 1990).

Peptide segments of the parental HER-2/neu protein with a motif with theoretical potential to bind to class II MHC molecules were identified herein. Locating potential T cell

epitopes was aided by computer analysis. A protein sequence analysis package, T Sites, that incorporates several computer algorithms designed to distinguish potential sites for T cell recognition was used (Feller and de la Cruz, *Nature* 349:720–721, 1991). Two searching algorithms were used: (1) the AMPHI algorithm described by Margalit (Feller and de la Cruz, *Nature* 349:720–721, 1991; Margalit et al., *J. Immunol.* 138:2213–2229, 1987) identified epitope motifs according to alpha-helical periodicity and amphipathicity; (2) the Rothbard and Taylor algorithm identified epitope motifs according to charge and polarity pattern (Rothbard and Taylor, *EMBO* 7:93–100, 1988). Segments with both motifs are most appropriate for binding to class II MHC molecules, with the caveat that each particular MHC molecule has a particular binding motif. Using this analysis, more than 40 potential T cell epitopes in the HER-2/neu protein corresponding to the AMPHI and the Rothbard motifs that would have the potential for binding to class II MHC molecules were identified (FIG. 1).

Peptides, each 15 amino acids in length, that encompass both the AMPHI and Rothbard motifs were constructed. The optimal peptide length for class II MHC binding depends upon the particular MHC molecule and may be shorter than 15 amino acids. However, class II MHC responses to exogenous peptides allow for endocytosis and intracellular processing of longer peptides. One of the synthetic peptides (p42–56), HLDMLRHLYQGCQVV (Seq. ID No. 30), is located in the extracellular domain and has 33% homology to epidermal growth factor receptor (EGFR). Two other synthetic peptides, SRLLGICLTSTVQLV (p783–797) (Seq. ID No. 45) and TLERPKTLSPGKNGV (p1166–1180) (Seq. ID No. 54)are both located in the intracellular domain and have 87% and 7% homology to EGFR respectively. The peptides as well as partially purified whole protein ($p185^{HER\text{-}2/neu}$) were used in subsequent defined experiments to detect CD4+ T cell proliferation responses (Section C. below).

B. p185(HER-2/neu) Protein can be Obtained and Purified from the Human Breast Adenocarcinoma Cell Line SKBR3

Purified p185 for T cell proliferation studies and antibody detection studies was obtained from the cell line SKBR3. SKBR3 has been reported on extensively in the literature as a commonly used standard cell line with increased HER-2/neu gene copy number and HER-2/neu protein overexpression. In one study, SKBR3 cells were found to contain a mean HER-2/neu oncogene copy number of 43 copies/cell compared with 2.5 copies/cell for MCF-7, a breast cancer cell line considered to be a standard cell line without HER-2/neu gene amplification (Kallionieme et al., *Proc. Natl. Acad. Sci.* USA 89:5321–5325, 1992). SKBR3 is reported to be one of the highest known expressors of $p185^{HER\text{-}2/neu}$ protein by immunohistochemistry, 4+ compared to 1+ in MCF-7 (Kerns et al., *J. Histochem. & Cytochem.* 38:1823–1830, 1990). The same HER-2/neu bands as described in the literature were validated in the present experiments by Western analysis. Bands detected included p185, p105 (extracellular domain), and several smaller bands that presumably represent fragments of phosphorylated protein (Alper et al., *Cell Growth and Differentiation* 1:591–599, 990; Zabrecky et al., *J. Biol. Chem.* 266:1716–1720, 1991; Stern et al., *Mol. Cell. Biol.* 8:3969–3973, 1988).

The antibodies used for detecting the HER-2/neu protein immunoblotting were commercially prepared by Oncogene Science (Manhasset, N.Y.). The antibody most commonly used in the present experiments was c-neu Ab-3; derived by immunization of BALB/c mice with a peptide sequence, TAENPEYLGLDVPV (Seq. ID No. 65), from the carboxyl domain of human c-neu gene product, and fusion of mouse splenocytes with SP2/0 myeloma cells. A second antibody, c-neu AB-1, gave very faint bands when compared with c-neu Ab-3. This antibody was a polyclonal rabbit affinity purified antibody against the peptide sequence, LARLLDIDETEYAD (Seq. ID No. 66), from the kinase domain of the human c-neu gene product.

Transmembrane $p185^{HER\text{-}2/neu}$ protein was purified from the cell membrane fraction of SKBR3 by modifications of described methods for other membrane-associated proteins (Dhut et al, *Leukemia* 4:745–750, 1990; Mietzner et al., *J. Exp. Med.* 165:1041–1057, 1987). Three$\times 10^6$ SKBR3 cells were harvested and suspended in phosphate buffered saline (PBS) with the following protease inhibitors; 1 mM PMSF, 1 mM benzamidine, 5 μg/ml aprotinin. All procedures were done on ice or at 4° C. The cells were then disrupted by sonication at 75W for a total of 1 minute using a high intensity sonifier equipped with a microtip (Branson Instruments, Inc., Stamford, Conn.). The resulting suspension was then centrifuged for 1 hour at 35,000 rpm to sediment membranes from cytosolic fraction. The membrane pellet was washed in ice cold PBS with protease inhibitors and the cycle of sonication/centrifugation was repeated twice. All cytosolic (supernatant) and membranous fractions were tested for the presence of $p185^{HER\text{-}2/neu}$ by Western analysis. The protein was noted to be strongly concentrated in the membrane fraction.

Protein concentration of one of these enriched membrane pellets was determined to be 2625 μg/ml (Protein BioRad assay). $p185^{HER\text{-}2/neu}$ is an estimated 8% of membrane protein in SKBR3 (Leitzel et al., *J. Clin. Oncol.* 10:1436–1443, 1992); therefore, an estimated 210 μg of $p185^{HER\text{-}2/neu}$ were present in the membrane pellet from 3×10^6 SKBR3 cells.

If desired, the membrane preparation may be further enriched for $p185^{HER\text{-}2/neu}$ e.g., by immunoprecipitation. Briefly, 1 μg of c-neu 3 antibody and 15 μl protein A agarose were added to the sonicated membrane pellet. The mixture was incubated at 4° C. on a rocker for 24 hours. The immunoprecipitate was collected by centrifugation in a micro-centrifuge at 2500 rpm for 15 minutes at 4° C., and the resulting pellet was washed several times with PBS, 1% Trition X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate. Silver stain and Western analysis showed increased concentration of $p185^{HER\text{-}2/neu}$ protein and decreases in extraneous membrane proteins when compared to membrane enriched pellets alone.

C. CD4+ T Cells Reactive to p185(HER-2/neu) Protein can be Elicited from PBL of Normal Individuals by using an Assay Designed for Detecting Low Frequency Lymphocyte Precursors

Three assays were used for the detection of $CD4^+$ responses: a standard proliferation assay, a screening method for low frequency events, and a limiting dilution assay (LDA). Conventional proliferative assays are capable of readily detecting primed responses. The proliferative response stimulation index provides a rough correlation with precursor frequency of antigen-reactive T cells. Any specific proliferative response detected from PBL is considered to be a primed response.

To provide a more quantitative interpretation of $CD4^+$ T cell responses, the assay system developed for detecting low lymphocyte precursor frequency responses (described below) is used. This assay is simple and cost-effective. In circumstances in which more precision is needed, the precursor frequency is validated by limiting dilution assays (Bishop and Orosz, *Transplantation* 47:671–677, 1989).

Responses greater than detected in normal individuals are defined as a primed response and imply existent immunity. Low responses, detectable only by LDA conditions are considered to be unprimed responses. An absent response by LDA or a response lower than that defined by the normal population analysis is considered to be tolerance/anergy.

In general, primed $CD4^+$ T cell responses can be detected in conventional proliferative assays, whereas unprimed responses are not detectable in the same assays. Detection of small numbers of unprimed T cells is limited by confounding background thymidine uptake including the autologous mixed lymphocyte response (AMLR) to self MHC antigen plus responses to processed self serum proteins and exogenously added serum proteins.

To elicit and detect unprimed T cells, an assay system for low frequency responses based on Poisson sampling statistics was used (In: *Pinnacles*, Chiron Corporation, 1:1–2, 1991). This type of analysis applies specifically to low frequency events in that, if the precursor frequency is less than the number of cells in one replicate culture, many replicates are required to detect a statistically significant number of positives. Theoretically, the analysis will correct for autologous responses by setting up a known positive control (such as PHA or tetanus toxoid) and known negative control (no antigen) and evaluating all data points from lowest to highest irrespective of the experimental group to which they belong. A cutoff value is calculated based on the equation cutoff=M+(F+SD), where M=arithmetic mean, F=3.29, a factor from tables of standardized normal distribution chosen so not more than 0.1% of the "true negatives" of a normally distributed background will be above the cutoff, and SD=standard deviation. In this screening assay, wells above the cutoff are considered true positives that potentially contain a lymphocyte that is specifically proliferating to the antigen of interest. Although estimations of lymphocyte precursor frequency is possible using this method, precise determination requires formal LDA analysis.

Analysis of PBL from normal individuals for HER-2/neu peptide and protein-specific T cells revealed the presence of a low level frequency of proliferative responses. A representative assay is described in FIG. 2. Seven normal subjects were analyzed, 4 males and 3 females. Of the seven individuals evaluated, 57% had a response to whole protein and 29% had a response to at least one individual peptide. The two individuals that responded to peptide also had responses to parental protein. Three males and one female had detectable responses to the whole protein. Two males responded to one of the four peptides. Similar methods can be used to elicit HER-2/neu reactive T cells from patients with HER-2/neu positive malignancies, but no prior priming in vivo. Alternatively, the methods can be used to assess the efficacy of priming to HER-2/neu in vivo and the procurement of immune T cells to be expanded for therapy.

D. CD4+ T Cells Reactive to p185 HER-2/neu) and Peptide can be Detected in the Peripheral Blood of Patients with HER-2/neu Positive Breast Cancer in Levels Consistent with a Primed Response

Four breast cancer patients with known HER-2/neu tumor status have been evaluated in a standard proliferation assay. Three patients had tumors which overexpressed the HER-2/neu protein. Proliferation to antigen was consistent with a primed response (FIG. 3) (i.e., proliferation was detectable in a standard proliferation assay with a Stimulation Index (S.I.) greater than 2). One patient was HER-2/neu negative and had a response towards intact HER-2/neu, but no response to HER-2/neu-derived peptides. The patients tested and chosen had different stages of disease and were in different stages of treatment. Five normal individuals' responses were analyzed in the same fashion, and none had an S.I. greater than 2 to any HER-2/neu protein or peptide (Table 1).

TABLE 1

(a) Breast Cancer patients

Patient	HER-2/neu Status	Tetanus Toxoid	p185	p42–56	p783–797	p1166–1180
A	Negative	52	13	2	2	2
B	Positive	36	<2	26	19	2
C	Positive	7	4	<2	<2	<2
D	Positive	10	4	4	5	<2

(b) Normal Individuals

Normal	Tetanus Toxoid	p185	p42–56	p783–797	p1166–1180
1	6	2	2	<2	<2
2	7	<2	2	<2	<2
3	7	<2	<2	<2	<2
4	10	2	ND	ND	<2
5	11	2	ND	ND	2

Example 2

$CD8^+$ CTL Specific for HER-2/neu Peptides Can Be Generated from PBL of Normal individuals by Primary In Vitro Immunization to Synthetic Peptides Derived from the Normal Amino Acid Sequence of p185(HER-2/neu) Protein

A. p185(HER-2/neu) Protein Contains Multiple Segments with an Amino Acid Sequence Motif Appropriate for Binding the Class I MHC Molecule HLA-A2.1

$CD8^+$ T cells recognize peptide bound to class I MHC molecules. In general, peptide determinants are derived from endogenously synthesized proteins. The rules which determine the ability of a protein to be processed and complexed with class I MHC molecules are not completely understood. Recently, however, it has been determined that peptides binding to particular MHC molecules share discernible sequence motifs (Falk et al., *Nature* 351:290–296, 1991). A peptide motif for binding in the groove of HLA-A2.1 has been defined by Edman degradation of peptides stripped from HLA-A2.1 molecules of a cultured cell line (Table 2, from Falk et al., supra). The method identified the typical or average HLA-A2.1 binding peptide as being 9 amino acids in length with dominant anchor residues occurring at positions 2 (L) and 9 (V). Commonly occurring strong binding residues have been identified at positions 2 (M), 4 (E,K), 6 (V), and 8 (K). The identified motif represents the average of many binding peptides.

TABLE 2

The HLA-A2.1 Restricted Motif

	Amino Acid Position									Point Assignment
	1	2	3	4	5	6	7	8	9	
Dominant Binding Anchor Residue		L							V	+3
Strong Binding		M		E		V		K		+2

TABLE 2-continued

The HLA-A2.1 Restricted Motif

	Amino Acid Position									Point Assignment
	1	2	3	4	5	6	7	8	9	
Residue				K						
Weak Binding Residue	I L F K M Y		A Y F P M S	G P D T	I K Y N G V H	I L T	A Y H	E S	L	+1

The derived peptide motif as currently defined is not particularly stringent. Some HLA-A2.1 binding peptides do not contain both dominant anchor residues and the amino acids flanking the dominant anchor residues play major roles in allowing or disallowing binding. Not every peptide with the current described binding motif will bind, and some peptides without the motif will bind. However, the current motif is valid enough to allow identification of some peptides capable of binding.

According to the current motif, the $p185^{HER\text{-}2/neu}$ protein contains a substantial number of peptides with amino acid sequences possibly appropriate for binding to the class I MHC antigen HLA-A2.1. Evaluation of the 1255 aa structure of $p185^{HER\text{-}2/neu}$ revealed at least 19 peptide segments of 9 aa in length that contained at least one of the dominant anchor residues. Of note, the current HLA-A2.1 motif places 6 amino acids between the dominant anchor amino acids at residues **2** and **9**. Recent studies show that alterations in secondary structure of peptides can sometimes allow for additional intervening residues, and thus longer binding peptides. In the present experiment, 9-mer peptides were evaluated. The 10 peptides with both dominant residues were considered. The arbitrary scoring system awarded +3 for a dominant anchor residue, +2 for a strong binding residue, and +1 for a weak binding residue. Emphasis was placed on presence or absence of dominant anchor residues as they appear to be of prime importance for peptide binding to HLA-A2 (Parker et al., *J. Immunol.* 148:3580–3587, 1992). Four peptides were synthesized (Table 3). One is located in the extracellular domain of the protein and three are located in the intracellular domain. Homology to EGFR ranges from 11% to 89% (Bargmann et al., *Nature* 319:226–230, 1986).

TABLE 3

$p185^{HER\text{-}2/neu}$ Peptides Constructed for Binding in HLA-A2.1 Motif

$p185^{HER\text{-}2/neu}$ Peptides	Amino Acid Position 1 2 3 4 5 6 7 8 9	Score	Location	Homology to EGFR
$p48–56^{HER\text{-}2/neu}$ (Seq. ID No. 1)	H L Y Q G C Q V V	8*	Extracellular	33%
$p789–797^{HER\text{-}2/neu}$ (Seq. ID No. 7)	C L T S T V Q L V	9*	Intracellular	89%
$p851–859^{HER\text{-}2/neu}$ (Seq. ID No. 9)	V L V K S P N H V	9*	Intracellular	78%
$p1172–1180^{HER\text{-}2/neu}$ (Seq. ID No. 10)	T L S P G K N G V	9*	Intracellular	11%

*Peptide contains both dominant anchor residues

B. Four of Four Peptides with a Motif Theoretically Appropriate for Binding to HLA-A2.1 can be Shown to Actually Bind to HLA-A2.1 in a Class I MHC Molecule Stabilization Assay

Having identified and synthesized peptides with a theoretical likelihood of binding to HLA-A2.1, the constructed peptides were evaluated as to whether in fact they could bind, the sine quo non of cytotoxic T lymphocytes (CTL) generation. Of the four peptides constructed, all could be shown to bind to HLA-A2 in an assay utilizing the mutant cell line T2. T2 is a human T-B cell hybrid that has a large homozygous deletion within the MHC gene region (Riberdy and Cresswell, *J. Immunol.* 148:2586–2590, 1992; Trousdale et al., Nature 348:741–744, 1990; Spies et al., *Nature* 348:744–747, 1990). The use of T2 to determine HLA-A2.1 binding peptides has been well defined. T2 does not appropriately process endogenous antigen for presentation with class I MHC molecules. Consequently, cell surface expression of class I MHC molecules is markedly reduced. However, provision of exogenous peptides which bind to and stabilize class I MHC in the presence of B2 microglobulin results in increased levels of class I at cell surface which can be easily detected by immunofluorescent staining. T2 without exogenous peptide has low expression of HLA-A2 (30% –50%). When incubated with peptides able to bind A2, the level of class I MHC stabilizes on the cell surface and can be measured by immunofluorescent staining. Thus, the T2 line fails to present internal proteins in the class I pathway, but can bind exogenous peptides, providing that the exogenous peptides have the appropriate HLA-A2.1 binding motif.

In this experiment, 1×10^6 T2 cells were incubated with individual peptides at a concentration of 25 μg/ml for 18 hours at 37° C. Binding of peptides to HLA-A2 was determined by immunofluorescent staining with a mouse monoclonal HLA-A2 antibody followed by rabbit antimouse IgG-FITC conjugate. The peptides which bound HLA-A2 increased class I surface expression to 60% –85% (10–15 percentage points over baseline).

C. $CD8^+$ CTL Specific for HER-2/neu p48–56 and p789–797 can be Generated by Primary in Vitro Immunization

In general, detection of T cell responses in vitro implies prior priming has occurred in vivo. It has been difficult and rare to generate CTL in vitro from unprimed populations.

Conditions for detecting immunity to standard recall antigens were used and no peptide-specific CTL could be detected. A set of conditions were derived which have allowed priming to 4 of 4 of the binding peptides tested to date. The conditions were derived by empiric experimentation but are consistent with the current paradigm. Conditions include: (1) large numbers of T cells; (2) a concurrent stimulated primed $CD4^+$ T cell response; (3) IL-2 added late to culture in very small amounts; and (4) multiple restimulations.

Initial experiments examined response to p48–56 which is normally present in the extracellular domain and p789–797 which is normally present in the intracellular domain, both of which were found to bind to HLA-A2.1. Four of four peptides with a motif theoretically appropriate for binding to HLA-A2.1 are shown to actually bind to HLA-A2.1 in a class I MHC molecule stabilization assay (Table 4). T2 cells were incubated for 18 hours with the depicted synthetic $p185^{HER\text{-}2/neu}$ peptides. Cells were then washed and stained with antihuman HLA-A2 antibody (3%), a second step FITC-conjugated antibody (3%) was then added. The % increase of class I on cell surface as measured by increased fluorescent intensity of cells incubated with peptide compared to cells incubated in medium alone is indicated.

TABLE 4

$p185^{HER-2/neu}$ Peptides	% Increase of class I stabilization on T2
p48–56	20%
p789–797	20%
p851–859	12%
p1172–1180	10%

After leukapheresis of a normal homozygous HLA-A2 individual, bulk cultures of lymphocytes (3×10^7) were incubated with peptide in a concentration of 10 μg peptide/mi. An individual homozygous for HLA-A2.1 was used on the presumption that a double dose of the MHC/peptide complex would allow more effective priming. Large numbers of lymphocytes were used to overcome the presumed low frequency of precursors. Generation of $CD8^+$ CTL responses has long been known to require concurrent stimulation of $CD4^+$ T cell responses to provide help/amplification. Both peptides used were chosen for class I MHC binding, and presumably could not stimulate $CD4^+$ helper T cells. To provide T cell help, low concentrations (5 μg/ml) of tetanus toxoid were added to culture along with peptide. So as not to overwhelm or dominate the culture with the tetanus toxoid response, titrations of tetanus toxoid had previously been assessed in a standard proliferation assay with the donor's lymphocytes and the concentration of tetanus toxoid that provided the lowest detectable stimulation index was used.

Low doses of IL-2 added late to culture were used to maintain lymphocyte proliferation. Within the present disclosure, standard conditions for expanding in vivo primed CTL following secondary sensitization in vitro usually have included IL-2 at 5–10 U/ml on day 2 of stimulation. Under primary in vitro immunization conditions, similar concentrations of IL-2 induced expansion of non-specifically lytic NK and T cells, presumably due to the predominance of NK cells and AMLR responsive cells relative to peptide-specific CTL. For in vitro priming, the T cell culture received no IL-2 for the first 10 days, with only 1 unit/ml administered on day +2 after the second IVS. Thereafter, IL-2 at 2 U/ml could be administered on day +2 and day +4 of the 7 day stimulation cycle. T cells were stimulated with peptide on irradiated PBL as APC every 7 days. Evaluation for specific lytic function was performed after the fourth IVS and revealed specific lytic activity but substantial non-specific lytic NK and T cell activity. Routine ^{51}Cr release assay performed after the tenth IVS (FIG. **4**) revealed greater than 50% lysis for both bulk T cell lines. Lysis against control targets of K562 and Daudi was less than 2%.

Example 3

Antibodies Directed Against HER-2/neu Protein Can Be Detected in the Sera of Patients with Breast Cancer

A. Antibodies Directed against p185(HER-2/neu) Protein and $p10^5$ (HER-2/ncu) Extracellular Domain were Detected in the Sera of some Breast Cancer Patients

The sera of 20 patients with breast cancer were analyzed. The 20 patients were participants from the Fred Hutchinson Cancer Research Center, Division of Epidemiology WISH study. The patient population consisted of women recently diagnosed with breast cancer, generally less than 3 months from surgery. Their age was less than 55 and their HER-2/neu tumor status was unknown. Anti-p185 antibody was found in 55% of the group as evidenced by bands corresponding to the positive control.

Antibody analysis was based on a modification of standard Western blotting techniques (Laemmli, *Nature* 227:680–685, 1970; Burnett, *Anal. Biochem.* 112:195–203, 1981)., A 7.5% SDS polyacrylamide gel was poured with a single 12 cm long comb in the stacking gel to create a "trough." Two immunoprecipitated SKBR3 membrane preparations, described above, were dissolved in loading buffer and layered across the trough. The gel was then run in standard fashion resulting in a band of equally distributed proteins across the gel. The protein was transferred to nitrocellulose (Amersham Hybond) for subsequent immunoblotting and development by chemiluminescence methods (Amersham ECL). Once protein transfer was complete, the nitrocellulose was cut lengthwise into 25 equal strips and placed in a 25 well incubation tray. The nitrocellulose strips were then blocked with Tris buffered saline and 1% bovine serum albumin (TBS BSA) for 1 hour. This allows for analysis of 23 patients with 2 control strips. Patient sera is used as primary antibody, and after blocking, the strips are incubated for 24 hours at 4° C. with sera diluted 1:200 and 1:400 in TBS BSA. The second antibody is a goat antihuman HRP conjugate which will interact with the chemiluminescent developing reagent (Amersham ECL) resulting in light emission which can be photographed. A control strip is developed with c-neu Ab3 antibody previously described in a similar fashion with this assay both IgA and IgG antibody specific for p185 were detected. Patient sera identified the same p185 band (FIG. **5**) as did the known HER-2/neu-specific antibody, providing evidence that some patients have existent antibody immunity to HER-2/neu.

To validate these responses patient sera was tested against a murine cell line (NIH 3T3) that had been transfected with HER-2/neu cDNA. As a negative control, untransfected cells were used. Membrane preparations were prepared from the two cell lines and patient sera was used as primary antibody as previously described. The patient sera identified the same p185 band as did the known HER-2/neu-specific antibody. That band was present in the cells that contained HER-2/neu, but undetectable in the cells that did not contain HER-2/neu (FIG. **6**).

Recombinant proteins of the extracellular and intracellular domain portions of HER-2/neu were obtained. The extracellular protein (110 kD) and intracellular protein (75 kD) were resolved on a 7.5% SDS-PAG gel and incubated with patient sera as primary antibody as previously described. The sera identified both proteins proving that some patients have antibodies directed to both the extracellular and intracellular domain of the HER-2/neu protein (FIG. **7**).

B. Seven Normal Individuals Showed no Evidence of Antibody to HER-2/neu Protein

In studies to determine the extent to which detection of antibody to HER-2/neu is specific for malignancy, sera from seven normal individuals was obtained and analyzed in identical fashion as described above. There was no evidence of antibodies directed toward any HER-2/neu protein.

C. The Sera of Three Patients with Known HER-2/neu Positive Tumors Contained Antibodies against p185 and p105

Sera from breast cancer patients whose HER-2/neu tumor status is known was collected and analyzed to determine the extent to which antibody to HER-2/neu correlates with the presence of HER-2/neu-positive tumors. Three patients with overexpression of $p185^{HER-2/neu}$ protein in their primary

tumor were analyzed. Antibodies against p185 were detected in all three. The antibody detected in our studies was IgG. Immunoglobulin class switch from igM to IgG or IgA require T cell help often directed against different epitopes on the same protein molecule.

Example 4

Peptide Based Vaccines Elicit Immunity to HER-2/neu

A. Materials and Methods

1. Animals

Rats used in this study were Fischer strain 344 (CDF (F-344)/CrlBR) (Charles River Laboratories, Portage Mich.). Animals were maintained at the University of Washington Animal facilities under specific pathogen free conditions and routinely used for experimental studies between 3 and 4 months of age.

2. Antigens

Nine peptides were constructed, derived from the amino acid sequence of the rat neu protein. The peptides, 15–18 amino acids in length, were highly homologous to the human HER-2/neu peptide sequence. These peptides were chosen based on an increased probability of interaction with human Class II MHC molecules. This theoretical potential was evaluated by the use of a protein sequence analysis package, TSites, that incorporates several computer algorithms designed to distinguish potential sites for T cell recognition (Feller and de la Cruz, *Nature* 349:720–721, 1991). Several peptides identified from the rat sequence were predicted to have potential for class II interaction with both human and murine MHC. Nine peptides were chosen for immunization of the rats (Table 5). Eight of the nine were in areas of 100% homology with human neu. The remaining peptide had greater than 80% homology with human neu (Yamamoto et al., *Nature* 319:230–234, 1986). The peptides were synthesized and purified by H. Zabrowski (University of Washington, Seattle, Wash.), then dissolved in phosphate-buffered saline (PBS), pH 7.4, to give 2 mg/ml stock solutions. Prior to aliquoting, peptides were sterile filtered, then stored at −70° C.

TABLE 5

Peptides from the Rat neu Protein for Immunization			
Rat Sequence	Amino Acids	Protein Domain	Homology to Human neu
p45–59	HLDMLRHLYQGCQVV (Seq. ID No. 30)	ECD	100%
p98–112	PLQRLRIVRGTQLFE (Seq. ID No. 31)	ECD	100%
p323–337	NQEVTAEDGTQRCEK (Seq. ID No. 56)	ECD	100%
p332–349	TQRCEKCSKPCARVCYGL (Seq. ID No. 60)	ECD	100%
p433–447	RIIRGRILHDGAYSL (Seq. ID No. 67)	ECD	80%
p781–795	GVGSPYVSRLLGICL (Seq. ID No. 44)	ICD	100%
p788–802	SRLLGICLTSTVQLV (Seq. ID No. 45)	ICD	100%
p932–946	PAREIPDLLEKGERL (Seq. ID No. 49)	ICD	100%
p1171–1185	TLERPKTLSPGKNGV (Seq. ID No. 54)	ICD	100%

ECD = extracellular domain
ICD = intracellular domain

3. Immunization

One group of rats was immunized with a mixture of extracellular domain (ECD) peptides and one group with a mixture of intracellular domain (ICD) peptides. The final group received adjuvant alone. Peptides were administered at a final concentration of 100 μg each in a total volume of 200 μl. The animals underwent 3 immunizations each 14–16 days apart with either CFA or IFA as adjuvant (Sigma ImmunoChemicals, St. Louis, Mo.). 16 days after the third immunization sera was obtained for assessment of immune response.

4. Cell Lines

Two cell lines were used as a source of neu proteins. SKBR3, a human breast cancer cell line that is a marked overexpressor of HER-2/neu (American Type Culture Collection, Rockville, Md.), was maintained in culture in 10% fetal bovine serum (FBS) (Gemini Bioproducts, Inc., Calabasas, Calif.) and RPMI. DHFR-G8, an NIH/3T3 cell line cotransfected with cneu-p and pSV2-DHFR (American Type Culture Collection, Rockville, Md.), was used as a source of non-transforming rat neu protein (Bernards et al., *Proc. Natl. Acad. Sci.* USA 84:6854–6858, 1987). This cell line was maintained in 10% FBS and Dulbecco's modified Eagle's medium with 4.5 g/L glucose. DHFR-G8 cells were passaged through the same medium supplemented with 0.3 μM methotrexate at every third passage to maintain the neu transfectant.

5. Preparation of Cell Lysates

Lysates of both SKBR3 and DHFR-G8 were prepared and used as a source of protein for both ELISA and immunoprecipitation studies. Briefly, a lysis buffer consisting of tris base, sodium chloride and Triton-X (1%) pH 7.5 was prepared. Protease inhibitors were added; aprotinin (1 Mg/ml), benzamidine (1 mM) and PMSF (1 mM). 1 ml of the lysis buffer was used to suspend 10^7 cells. The cells were vortexed for 15 seconds every 10 minutes for an hour until disrupted. All procedures were performed on ice in a 4° C. cold room. After disruption the cells were microfuged at 4° C. for 20 minutes. Supernatant was removed from cell debris and stored in small aliquots at −70° C. until used. Presence of human and rat neu in the lysates was documented by Western blot analysis.

6. ELISA for Rat Antibody Responses

96 well Immulon 4 plates (Baxter SP, Redmond, Wash.: Dynatech Laboratories) were incubated overnight at 4° C. with an IgG2a murine monoclonal antibody directed against rat neu (kindly provided by Dr. M. Green) at a concentration of 10 μg antibody per ml. After incubation, all wells were blocked with PBS and 1% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, Mo.), 100 μl/well for 4 hours at room temperature. The plate was washed with PBS/0.5% Tween and protein was added. Rows of wells were coated with alternating PBS/1% BSA and DHFR-G8 lysate (10^8 cells/20 ml PBS), 50 μl/well, overnight at 4° C. After washing, the plate was incubated with rat sera at the following dilutions: 1:25, 1:50, 1:100, 1:200. The sera was diluted in PBS/1% BSA/1% FBS/25 μg/ml mouse igG/0.01% NaN_3 and then serially into PBS/1% BSA. 50 μl of diluted sera was added/well and incubated 1 hour at room temperature. Sheep anti-rat Ig horseradish peroxidase (HRP) was added to the wells at a 1:7,500 dilution in PBS/1% BSA and incubated for 45 minutes at room temperature (Amersham Co., Arlington Heights, Ill.). isotype assays were performed similarly with rabbit anti-rat IgG and sheep anti-rat IgM HRP antibodies as the second step antibody at a concentration of 1:5000 (Serotec Ltd., Oxford, England).

Control wells consisting of varying dilutions of c-neu-Ab-1, a rabbit polyclonal antibody directed against the kinase portion of human neu which also has reactivity to rat neu (Oncogene Science, Uniondale, N.Y.), were used as a positive control. These wells received a second step antibody of goat-anti rabbit HRP at a 1:5000 dilution (Amersham Co.). Following the final wash, TMB (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) developing reagent was added. Color reaction was read at an optical density of 640 nm until the positive control wells reached 0.3 OD. The reaction was stopped with 1N HCl and the optical density was read at 450 nm. The OD of each serum dilution was calculated as the OD of the neu coated wells minus the OD of the PBS/1% BSA coated wells. A pool of 5 normal rat sera was run on each plate as a negative control.

7. Immunoprecipitation

Experimental rat sera was used to inununoprecipitate human neu from the SKBR3 cell line and rat neu from the DHFR-G8 cell line. A commercially prepared IgG1 mouse monoclonal antibody, c-neu-Ab-3, which cross reacts with both human and rat neu, was used as the positive control antibody in the immunoprecipitation (Oncogene Science). Sera from 5 pooled normal rats and 2 rats immunized with adjuvant alone and no peptide antigens were used as 2 negative controls. 1 ml of DHFR-G8 or SKBR3 lysate was incubated with 75 µl of rat sera or 10 µl (1 µg) of neu specific monoclonal antibody and 15 µl of protein A+G (Oncogene Science). The solution was rocked gently overnight at 4° C. After this incubation, the agarose was pelleted and washed twice in a tris HCl/EDTA buffer (1M Tris HCl pH 7.5, 0.25M EDTA, and 5M NaCl), then twice in the same buffer with NP-40 added to a 0.5% concentration. The immunoprecipitates were analyzed by Western blot as described above using c-neu-Ab-1 (Oncogene Science) as the primary antibody. This antibody is a neu specific polyclonal rabbit antibody which cross reacts with both human and rat neu.

8. ELISA for Peptide Epitope Analysis

96 well Immulon 4 plates (Dynatech Laboratories) were incubated overnight at 4° C. with peptides at a concentration of 10 µg/well diluted in PBS alternating with rows of PBS/1% BSA. After incubation, all wells were blocked with PBS/1% BSA, 100 µl/well for 4 hours at room temperature. The plate was washed with PBS/0.5% Tween. After washing, the plate was incubated with rat sera at the following dilutions: 1:50 and 1:100. The sera was diluted in PBS/1% BSA/1% FBS/25 µg/ml mouse IgG/0.01% NaN_3 and then serially into PBS/1% BSA. 50 µl of diluted sera was added/well and incubated 1 hour at room temperature. Sheep anti-rat HRP was added to the wells at a 1:7,500 dilution in PBS/1% BSA and incubated for 45 minutes at room temperature. Following the final wash, the TMB developing reagent was added. Color reaction was read at an optical density of 640 nm until the reading on the most reactive well reached 0.30D. The reaction was stopped with 1N HCl and the optical density was read at 450 nm. The OD of each serum dilution was calculated as the OD of the peptide coated wells minus the OD of the PBS/1% BSA coated wells. A pool of 5 normal rat sera was run with each peptide at the same dilutions as the experimental sera as a negative control.

9. Western Blot Analysis for Rat Antibody Responses

Immunoprecipitates of SKBR3 and DHFR-G8 were used as a source of human and rat neu proteins in the Western assays. Recombinant human ECD and ICD (kindly provided by Drs. B. Groner and N. Lydon) were used to evaluate antibody responses to the neu domains. 7.5% polyacrylamide gels were electrophoresed in the Pharmacia Phast System (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). After transfer to nitrocellulose (Hybond-C, Amersham Co.) the neu proteins were identified by immunoblot in a similar manner. All control blots were developed by using the IgG1 mouse monoclonal primary antibody, c-neu-Ab-3 (Oncogene Science). This antibody cross reacts with both rat and human neu. The primary antibody was used in a 1:1000 dilution with tris-buffered saline/1% BSA/0.1% Nonidet P-40. A polyclonal rabbit antimouse HRP-conjugated second antibody (Amersham Co.) was used in a 1:10,000 dilution. The blot was then developed using a chemiluminescent reaction (Amersham ECL). Identically run experimental blots were analyzed with rat sera as primary antibody. The sera were used in a 1:500 dilution with tris-buffered saline/1% BSA/0.1% Nonidet P-40 in an overnight incubation with the blot a 4° C. Secondary antibody, goat-anti rat HRP conjugate (Amersham Co.) was used at a 1:5000 dilution. The blots were developed with ECL detection reagents and exposed to Hyperfilm—ECL (Amersham Co.). The film was developed and examined for reaction to human and rat neu as well as the ICD and ECD domains of the protein. Sera from 5 pooled normal rats and 2 rats immunized with adjuvant alone and no peptide antigens were used as 2 negative controls.

10. T Cell Proliferation Assays

For analysis of neu peptide specific responses: Fresh spleen or lymph node cells were harvested by mechanical disruption and passage through wire mesh and washed. 2×10^5 spleen cells/well and 1×10^5 lymph node cells/well were plated into 96-well round bottom microtiter plates Corning, Corning, N.Y.) with 6 replicates per experimental group. The media used consisted of EHAA 120 (Biofluids) with L-glutamine, penicillin/streptomycin, 2-mercaptoethanol, and 5% FBS. Cells were incubated with 25 µg/ml of the various peptides. The group incubated with the peptide mix received 25 µg of each of the peptides. After 4 days, wells were pulsed with 1 µCi of [^{3}H]thymidine for 6–8 hours and counted. Data is expressed as a stimulation index (SI) which is defined as the mean of the experimental wells divided by the mean of the control wells (no antigen). For analysis of neu protein specific responses: Spleen or lymph node cells were cultured for 3 in vitro stimulations. At the time of analysis 1×10^5 cultured spleen or lymph node T cells were plated into 96 well microtiter plates as described above. Cells were incubated with 1 µg/ml immunoaffinity column purified rat neu (from DHFR-G8 cells as the source of rat neu). After 4 days, wells were pulsed with 1 µCi of [$_3$H]thymidine for 6–8 hours and counted. Data is expressed as a stimulation index which is defined as the mean of the experimental wells divided by the mean of the control wells (no antigen).

11. Rat T Cell Culture

Spleen and lymph nodes from immunized rats were harvested into single cell suspensions. PBMC were isolated by Ficoll/Hypaque density gradient centrifugation (Histopaque-1083, Sigma Diagnostics, St. Louis, Mo.). Cells were washed and resuspended in bulk culture of 3×10^7 cells in 6 well plates. The media used consisted of EHAA 120 (Biofluids) with L-glutamine, penicillin/streptomycin, 2-mercaptoethanol, and 10% FBS. A mix of the immunizing peptides were added directly to culture at a concentration of 10 µg/ml of each peptide. The cultures were restimulated on the peptide mix every 14 days with syngeneic spleen that had been preincubated with the peptide mix for 2 hours, irradiated to 1000 rads, and then washed. Stimulator to effector ratio was 1:1 in each culture. After the second week

in culture, media was supplemented with 50% Con A conditioned media. At the end of 3 in vitro stimulations, cells were >98% CD3+.

B. Rats Immunized with Peptides Derived from the ICD Portion of Rat Neu Protein Develop Antibody Responses to Neu Protein

Rats were immunized with mixtures of either 4 ICD peptides or 5 ECD peptides. Following the third immunization, serum and T cells from immunized rats were assessed for immunity to neu peptides and protein. Initial experiments assessed rats immunized with ICD peptides for antibody responses to whole neu protein. Serum antibody responses were analyzed by ELISA (FIG. 8). The results demonstrate that immunization to ICD peptides elicited antibody to whole neu protein. Sera was analyzed at 1:25, 1:50, 1:100, and 1:200 dilution. Results at the 1:25 dilution are depicted (FIG. 8) . Neu specific antibody responses titered rapidly and at a 1:200 dilution the experimental sera demonstrated the same level of response as control. Isotype analysis revealed that the antibody responses were predominantly IgG (data not shown

C. Rats Immunized with Peptides Derived from the ECD Portion of Rat Neu Protein Develop Antibody Responses to Neu Protein

Immunizations with ECD peptides were performed in an identical fashion as with ICD peptides. ELISA performed on sera from rats immunized with ECD peptides revealed the generation of antibody responses to whole neu protein (FIG. 9). The responses were equivalent to responses elicited by immunization with ICD peptides. These responses were predominantly of the IgG subtype (data not shown).

D. Epitope Analysis of ICD Antibody Responses Demonstrates Dominant B Cell Epitopes as Well as "Determinant Spreading" between Domains

Mixtures of peptides had been used above for immunization. To determine which peptides in the mixture were the predominant B cell epitopes, sera from rats immunized with ICD peptides was analyzed by ELISA for responses to individual peptides. Responses to both ICD and ECD peptides were evaluated with the presumption that responses to the ECD peptides would be non-existent. Results (FIG. 10) revealed different responses in each rat. All rats had marked antibody responses to the overlapping p781 and p788 ICD peptides, although the relative levels of responses varied between animals. Responses to p932 and p1171 were observed, but were relatively weak. Surprisingly, rats immunized to the mixture of ICD peptides displayed significant antibody responses to ECD peptides. Responses in individual rats varied. Rat 2.2 had substantial responses to all five ECD peptides evaluated. Rats 2.1 and 2.3 had weaker responses. Thus, immunization to ICD peptides elicited antibody responses to ICD peptides as well as "determinant spreading" with the generation of antibody responses to the ECD portion of the molecule. Rats immunized with adjuvant alone did not develop T cell responses to any tested peptide.

E. Epitope Analysis of ECD Antibody Responses Demonstrates Dominant B Cell Epitopes

Determination of the dominant B cell epitopes in ECD peptide immunized animals was performed in an identical fashion. Again, the relative responses to individual peptides differed between each animal. Rats immunized with ECD peptides developed substantial responses to p45, p332, and p433 and minimal responses to p98 and p323 (FIG. 11). The dominant epitope was p45 in rats 1.1 and 1.3, but was p433 in rat 1.2. As with immunization to ECD peptides, determinant spreading was observed. All rats developed antibody to p788 in the ICD and rats 1.1 and 1.2 responded to p1171. The magnitude and extent of "determinant spreading" appeared to be less in the animals immunized with the ECD peptides than those immunized with the ICD peptides. However, only a limited number of potential epitopes were examined.

F. Antibodies Elicited by Immunization to Either ICD or ECD Peptides are Specific for and can Immunoprecipitate both Rat Neu Protein and Human HER-2/neu Protein

The above experiments showed that immunization to neu peptides could elicit antibody responses to whole rat protein and peptides, as determined by ELISA. Verification of the antibody responses to protein observed by ELISA was performed by assessing the ability of immune sera to immunoprecipitate rat neu protein from lysates of DHFRG-8, an NIH-3T3 cell line transfected with non-transforming rat neu. Results showed that sera from rats immunized with either ECD or ICD peptides could immunoprecipitate rat neu (FIG. 12, Panel A).

The immunizing rat neu peptides were homologous with the human HER-2/neu protein sequence. Thus, the anti-peptide antibodies elicited should be reactive to both rat and human peptides. To determine whether the antibodies elicited were also specific for human HER-2/neu protein, experiments evaluated the ability of sera from peptide immunized rats to immunoprecipitate HER-2/neu from lysates of SKBR3, a human breast cancer cell line that overexpresses HER-2/neu. Sera from all rats immunized with ICD or ECD peptides could immunoprecipitate HER-2/neu protein while the control sera did not (FIG. 12, Panel B).

G. B cell Epitopes that are Cross Reactive between Human and Rat Neu are Present in both Domains of the Protein

Antibody elicited by immunization to ICD and ECD peptides immunoprecipitated both rat and human neu protein. To further evaluate the protein domains recognized, sera from rats immunized with ICD and ECD peptides was evaluated by Western analysis for reactivity against human recombinant ECD and ICD as well as whole human and rat neu immunoprecipitated protein. Sera from animals inmmunized with either ICD or ECD peptides recognized both domains and whole protein from both species (FIG. 13). Control animals had no evidence of antibodies directed against either domain. These results verify not only the phenomenon of "determinant spreading" suggested in the peptide epitope analysis, but also demonstrate human and rat cross reactive epitopes in both domains.

H. Immunization of Rats with ICD Peptides Elicits Neu Peptide-Specific T Cell Responses

The above-detected antibody responses were IgG implying that T cell help was present and operative in immunoglobulin class switch. Spleen and lymph nodes cells were evaluated for proliferative responses to the immunizing peptides. Proliferative T cell responses to the immunizing peptides were observed, but the relative responses between individual rats were varied (FIG. 14). A stimulation index of >2 was arbitrarily chosen as the cut off of significance. Rat 2.1 did not have any proliferative response greater than SI of 2 to the mixture of immunizing ICD peptides or to individual peptides. Rats 2.2 and 2.3 had SI>2 to the mixture of ICD peptides with the dominant response to p1171 in both rats.

I. Immunization of Rats with ICD Peptides Elicits Neu Protein-Specific T Cell Responses

Peptide specific T cell lines were derived by repeated in vitro stimulation of spleen cells from peptide immunized mice by a mixture of the immunizing peptides. After 40 days the cultured cells were greater than 98% CD3+. The cultured T cells from 2 of the 3 immunized rats demonstrated

substantial responses to protein with SIs of 9 and 16 (FIG. 15). The SI from the third rat was >2. No responses to control protein were observed.

J. Immunization of Rats with ECD Peptides Elicits only Weak Peptide-Specific T cell Responses

A similar analysis was performed with T cells derived from animals immunized with the ECD peptides. Unlike the responses observed from the animals immunized with the mixture of ICD peptides, animals immunized with ECD peptides exhibited only weak proliferative responses to the mixture of ECD peptides as well as to individual peptides (FIG. 16). Only one of three rats displayed SI of 2.0 or greater to peptides.

K. Immunization of Rats with ECD Peptides Elicits Weak, but Positive Responses to Neu Protein

Both splenic and lymph node T cells derived from ECD peptide immunized rats were analyzed for responses to rat neu protein (FIG. 17). Splenic T cells exhibited low level responses, whereas responses were greater for lymph node derived T cells. Proliferative responses were not the same for all animals tested. The maximum SI for spleen derived T cell lines was 2.1, whereas the maximum SI for lymph node derived T cells was 3.

From the foregoing, it will be evident that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 68

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

His Leu Tyr Gln Gly Cys Gln Val Val
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Pro Leu Gln Pro Glu Gln Leu Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Pro Leu Thr Ser Ile Ile Ser Ala Val
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ile Leu Leu Val Val Val Leu Gly Val
1 5

-continued

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Leu Leu Val Val Val Leu Gly Val Val
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Arg Leu Leu Gln Glu Thr Glu Leu Val
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Cys Leu Thr Ser Thr Val Gln Leu Val
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Asp Leu Ala Ala Arg Asn Val Leu Val
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Val Leu Val Lys Ser Pro Asn His Val
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Thr Leu Ser Pro Gly Lys Asn Gly Val
1 5

(2) INFORMATION FOR SEQ ID NO:11:

-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Val Leu Gly Val Val Phe Gly Ile Leu
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Leu Ile Lys Arg Arg Gln Gln Lys Ile
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Lys Ile Pro Val Ala Ile Lys Val Leu
1 5

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Ile Leu Asp Glu Ala Tyr Val Met Ala
1 5

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Gln Leu Met Pro Tyr Gly Cys Leu Leu
1 5

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Gln Ile Ala Lys Gly Met Ser Tyr Leu
1 5

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids

-continued

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Leu Leu Asn Trp Cys Met Gln Ile Ala
1 5

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Arg Leu Val His Arg Asp Leu Ala Ala
1 5

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Asp Ile Asp Glu Thr Glu Tyr His Ala
1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Asp Leu Leu Glu Lys Gly Glu Arg Leu
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Thr Ile Asp Val Tyr Met Leu Met Val
1 5

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Met Ile Met Val Lys Cys Trp Met Ile
1 5

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

-continued

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Asp Leu Val Asp Ala Glu Glu Tyr Leu
1 5

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Gly Leu Glu Pro Ser Glu Glu Glu Ala
1 5

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Tyr Leu Thr Pro Gln Gly Gly Ala Ala
1 5

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Gln Leu Phe Glu Asp Asn Tyr Ala Leu
1 5

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Lys Ile Phe Gly Ser Leu Ala Phe Leu
1 5

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Ile Leu Asp Glu Ala Tyr Val Met Ala Gly Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid

-continued

(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Val Met Ala Gly Val Gly Ser Pro Tyr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:30:

His Leu Asp Met Leu Arg His Leu Tyr Gln Gly Cys Gln Val Val
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Pro Leu Gln Arg Leu Arg Ile Val Arg Gly Thr Gln Leu Phe Glu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Leu Arg Ser Leu Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Val Thr Tyr Asn Thr Asp Thr Phe Glu Ser Met Pro Asn Pro Glu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:34:

His Leu Arg Glu Val Arg Ala Val Thr Ser Ala Asn Ile Gln Glu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

-continued

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Val Arg Ala Val Thr Ser Ala Asn Ile Gln Glu Phe Ala Gly Cys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Asn Ile Gln Glu Phe Ala Gly Cys Lys Lys Ile Phe Gly Ser Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Gln Val Phe Glu Thr Leu Glu Glu Ile Thr Gly Tyr Leu Tyr Ile
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Gln Glu Cys Val Glu Glu Cys Arg Val Leu Gln Gly Leu Pro Arg
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Val Val Val Leu Gly Val Val Phe Gly Ile Leu Ile Lys Arg Arg
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:40:

Lys Tyr Thr Met Arg Arg Leu Leu Gln Glu Thr Glu Leu Val Glu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:41:

-continued

```
Gly Ala Met Pro Asn Gln Ala Gln Met Arg Ile Leu Lys Glu Thr
1               5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:42:

```
Val Lys Val Leu Gly Ser Gly Ala Phe Gly Thr Val Tyr Lys Gly
1               5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:43:

```
Ser Pro Lys Ala Asn Lys Glu Ile Leu Asp Glu Ala Tyr Val Met
1               5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:44:

```
Gly Val Gly Ser Pro Tyr Val Ser Arg Leu Leu Gly Ile Cys Leu
1               5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:45:

```
Ser Arg Leu Leu Gly Ile Cys Leu Thr Ser Thr Val Gln Leu Val
1               5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:46:

```
Gly Ser Gln Asp Leu Leu Asn Trp Cys Met Gln Ile Ala Lys Gly
1               5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:47:

```
Val Lys Ile Thr Asp Phe Gly Leu Ala Arg Leu Leu Asp Ile Asp
```

-continued

1 5 10 15

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:48:

Thr Val Trp Glu Leu Met Thr Phe Gly Ala Lys Pro Tyr Asp Gly
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:49:

Pro Ala Arg Glu Ile Pro Asp Leu Leu Glu Lys Gly Glu Arg Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:50:

Arg Phe Arg Glu Leu Val Ser Glu Phe Ser Arg Met Ala Arg Asp
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:51:

Glu Asp Asp Asp Met Gly Asp Leu Val Asp Ala Glu Glu Tyr Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:52:

Gly Met Gly Ala Ala Lys Gly Leu Gln Ser Leu Pro Thr His Asp
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:53:

Thr Cys Ser Pro Gln Pro Glu Tyr Val Asn Gln Pro Asp Val Arg
1 5 10 15

-continued

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:54:

Thr Leu Glu Arg Pro Lys Thr Leu Ser Pro Gly Lys Asn Gly Val
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:55:

Gly Gly Ala Val Glu Asn Pro Glu Tyr Leu Thr Pro Gln Gly Gly
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:56:

Asn Gln Glu Val Thr Ala Glu Asp Gly Thr Gln Arg Cys Glu Lys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:57:

Gln Val Ile Arg Gly Arg Ile Leu His Asn Gly Ala Tyr Ser Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:58:

Leu Gln Val Phe Glu Thr Leu Glu Glu Ile Thr Gly Tyr Leu Tyr
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:59:

Ala Ser Pro Leu Thr Ser Ile Ile Ser Ala Val Val Gly Ile Leu

-continued

1 5 10 15

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:60:

Thr Gln Arg Cys Glu Lys Cys Ser Lys Pro Cys Ala Arg Val Cys Tyr
1 5 10 15

Gly Leu

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:61:

Arg Leu Arg Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr Ala
1 5 10 15

Leu

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:62:

Lys Ile Phe Gly Ser Leu Ala Phe Leu Pro Glu Ser Phe Asp Gly Asp
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:63:

Arg Arg Leu Leu Gln Glu Thr Glu Leu Val Glu Pro Leu Thr Pro Ser
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:64:

Glu Leu Val Ser Glu Phe Ser Arg Met Ala Arg Asp Pro Gln
1 5 10

(2) INFORMATION FOR SEQ ID NO:65:

(i) SEQUENCE CHARACTERISTICS:

-continued

(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:65:

```
Thr Ala Glu Asn Pro Glu Tyr Leu Gly Leu Asp Val Pro Val
1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:66:

```
Leu Ala Arg Leu Leu Asp Ile Asp Glu Thr Glu Tyr Ala Asp
1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:67:

```
Arg Ile Ile Arg Gly Arg Ile Leu His Asp Gly Ala Tyr Ser Leu
1               5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1255 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:68:

```
Met Glu Leu Ala Ala Leu Cys Arg Trp Gly Leu Leu Leu Ala Leu Leu
1               5                  10                  15

Pro Pro Gly Ala Ala Ser Thr Gln Val Cys Thr Gly Thr Asp Met Lys
            20                  25                  30

Leu Arg Leu Pro Ala Ser Pro Glu Thr His Leu Asp Met Leu Arg His
        35                  40                  45

Leu Tyr Gln Gly Cys Gln Val Val Gln Gly Asn Leu Glu Leu Thr Tyr
    50                  55                  60

Leu Pro Thr Asn Ala Ser Leu Ser Phe Leu Gln Asp Ile Gln Glu Val
65                  70                  75                  80

Gln Gly Tyr Val Leu Ile Ala His Asn Gln Val Arg Gln Val Pro Leu
                85                  90                  95

Gln Arg Leu Arg Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr
            100                 105                 110

Ala Leu Ala Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro
        115                 120                 125

Val Thr Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser
    130                 135                 140

Leu Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln
145                 150                 155                 160

Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys Asn
                165                 170                 175

Asn Gln Leu Ala Leu Thr Leu Ile Asp Thr Asn Arg Ser Arg Ala Cys
```

-continued

180 185 190

His Pro Cys Ser Pro Met Cys Lys Gly Ser Arg Cys Trp Gly Glu Ser
195 200 205

Ser Glu Asp Cys Gln Ser Leu Thr Arg Thr Val Cys Ala Gly Gly Cys
210 215 220

Ala Arg Cys Lys Gly Pro Leu Pro Thr Asp Cys Cys His Glu Gln Cys
225 230 235 240

Ala Ala Gly Cys Thr Gly Pro Lys His Ser Asp Cys Leu Ala Cys Leu
245 250 255

His Phe Asn His Ser Gly Ile Cys Glu Leu His Cys Pro Ala Leu Val
260 265 270

Thr Tyr Asn Thr Asp Thr Phe Glu Ser Met Pro Asn Pro Glu Gly Arg
275 280 285

Tyr Thr Phe Gly Ala Ser Cys Val Thr Ala Cys Pro Tyr Asn Tyr Leu
290 295 300

Ser Thr Asp Val Gly Ser Cys Thr Leu Val Cys Pro Leu His Asn Gln
305 310 315 320

Glu Val Thr Ala Glu Asp Gly Thr Gln Arg Cys Glu Lys Cys Ser Lys
325 330 335

Pro Cys Ala Arg Val Cys Tyr Gly Leu Gly Met Glu His Leu Arg Glu
340 345 350

Val Arg Ala Val Thr Ser Ala Asn Ile Gln Glu Phe Ala Gly Cys Lys
355 360 365

Lys Ile Phe Gly Ser Leu Ala Phe Leu Pro Glu Ser Phe Asp Gly Asp
370 375 380

Pro Ala Ser Asn Thr Ala Pro Leu Gln Pro Glu Gln Leu Gln Val Phe
385 390 395 400

Glu Thr Leu Glu Glu Ile Thr Gly Tyr Leu Tyr Ile Ser Ala Trp Pro
405 410 415

Asp Ser Leu Pro Asp Leu Ser Val Phe Gln Asn Leu Gln Val Ile Arg
420 425 430

Gly Arg Ile Leu His Asn Gly Ala Tyr Ser Leu Thr Leu Gln Gly Leu
435 440 445

Gly Ile Ser Trp Leu Gly Leu Arg Ser Leu Arg Glu Leu Gly Ser Gly
450 455 460

Leu Ala Leu Ile His His Asn Thr His Leu Cys Phe Val His Thr Val
465 470 475 480

Pro Trp Asp Gln Leu Phe Arg Asn Pro His Gln Ala Leu Leu His Thr
485 490 495

Ala Asn Arg Pro Glu Asp Glu Cys Val Gly Glu Gly Leu Ala Cys His
500 505 510

Gln Leu Cys Ala Arg Cys His Cys Trp Gly Pro Gly Pro Thr Gln Cys
515 520 525

Val Asn Cys Ser Gln Phe Leu Arg Gly Gln Glu Cys Val Glu Glu Cys
530 535 540

Arg Val Leu Gln Gly Leu Pro Arg Glu Tyr Val Asn Ala Arg His Cys
545 550 555 560

Leu Pro Cys His Pro Glu Cys Gln Pro Gln Asn Gly Ser Val Thr Cys
565 570 575

Phe Gly Pro Glu Ala Asp Gln Cys Val Ala Cys Ala His Tyr Lys Asp
580 585 590

Pro Pro Phe Cys Val Ala Arg Cys Pro Ser Gly Val Lys Pro Asp Leu
595 600 605

-continued

Ser Tyr Met Pro Ile Trp Lys Phe Pro Asp Glu Glu Gly Ala Cys Gln
610 615 620

Pro Cys Pro Ile Asn Cys Thr His Ser Cys Val Asp Leu Asp Asp Lys
625 630 635 640

Gly Cys Pro Ala Glu Gln Arg Ala Ser Pro Leu Thr Ser Ile Ile Ser
645 650 655

Ala Val Val Gly Ile Leu Leu Val Val Val Leu Gly Val Val Phe Gly
660 665 670

Ile Leu Ile Lys Arg Arg Gln Gln Lys Ile Arg Lys Tyr Thr Met Arg
675 680 685

Arg Leu Leu Gln Glu Thr Glu Leu Val Glu Pro Leu Thr Pro Ser Gly
690 695 700

Ala Met Pro Asn Gln Ala Gln Met Arg Ile Leu Lys Glu Thr Glu Leu
705 710 715 720

Arg Lys Val Lys Val Leu Gly Ser Gly Ala Phe Gly Thr Val Tyr Lys
725 730 735

Gly Ile Trp Ile Pro Asp Gly Glu Asn Val Lys Ile Pro Val Ala Ile
740 745 750

Lys Val Leu Arg Glu Asn Thr Ser Pro Lys Ala Asn Lys Glu Ile Leu
755 760 765

Asp Glu Ala Tyr Val Met Ala Gly Val Gly Ser Pro Tyr Val Ser Arg
770 775 780

Leu Leu Gly Ile Cys Leu Thr Ser Thr Val Gln Leu Val Thr Gln Leu
785 790 795 800

Met Pro Tyr Gly Cys Leu Leu Asp His Val Arg Glu Asn Arg Gly Arg
805 810 815

Leu Gly Ser Gln Asp Leu Leu Asn Trp Cys Met Gln Ile Ala Lys Gly
820 825 830

Met Ser Tyr Leu Glu Asp Val Arg Leu Val His Arg Asp Leu Ala Ala
835 840 845

Arg Asn Val Leu Val Lys Ser Pro Asn His Val Lys Ile Thr Asp Phe
850 855 860

Gly Leu Ala Arg Leu Leu Asp Ile Asp Glu Thr Glu Tyr His Ala Asp
865 870 875 880

Gly Gly Lys Val Pro Ile Lys Trp Met Ala Leu Glu Ser Ile Leu Arg
885 890 895

Arg Arg Phe Thr His Gln Ser Asp Val Trp Ser Tyr Gly Val Thr Val
900 905 910

Trp Glu Leu Met Thr Phe Gly Ala Lys Pro Tyr Asp Gly Ile Pro Ala
915 920 925

Arg Glu Ile Pro Asp Leu Leu Glu Lys Gly Glu Arg Leu Pro Gln Pro
930 935 940

Pro Ile Cys Thr Ile Asp Val Tyr Met Ile Met Val Lys Cys Trp Met
945 950 955 960

Ile Asp Ser Glu Cys Arg Pro Arg Phe Arg Glu Leu Val Ser Glu Phe
965 970 975

Ser Arg Met Ala Arg Asp Pro Gln Arg Phe Val Val Ile Gln Asn Glu
980 985 990

Asp Leu Gly Pro Ala Ser Pro Leu Asp Ser Thr Phe Tyr Arg Ser Leu
995 1000 1005

Leu Glu Asp Asp Asp Met Gly Asp Leu Val Asp Ala Glu Glu Tyr Leu
1010 1015 1020

Val Pro Gln Gln Gly Phe Phe Cys Pro Asp Pro Ala Pro Gly Ala Gly
1025 1030 1035 1040

-continued

```
Gly Met Val His His Arg His Arg Ser Ser Ser Thr Arg Ser Gly Gly
                1045                1050                1055

Gly Asp Leu Thr Leu Gly Leu Glu Pro Ser Glu Glu Glu Ala Pro Arg
            1060                1065                1070

Ser Pro Leu Ala Pro Ser Glu Gly Ala Gly Ser Asp Val Phe Asp Gly
        1075                1080                1085

Asp Leu Gly Met Gly Ala Ala Lys Gly Leu Gln Ser Leu Pro Thr His
    1090                1095                1100

Asp Pro Ser Pro Leu Gln Arg Tyr Ser Glu Asp Pro Thr Val Pro Leu
1105                1110                1115                1120

Pro Ser Glu Thr Asp Gly Tyr Val Ala Pro Leu Thr Cys Ser Pro Gln
                1125                1130                1135

Pro Glu Tyr Val Asn Gln Pro Asp Val Arg Pro Gln Pro Pro Ser Pro
            1140                1145                1150

Arg Glu Gly Pro Leu Pro Ala Ala Arg Pro Ala Gly Ala Thr Leu Glu
        1155                1160                1165

Arg Pro Lys Thr Leu Ser Pro Gly Lys Asn Gly Val Val Lys Asp Val
    1170                1175                1180

Phe Ala Phe Gly Gly Ala Val Glu Asn Pro Glu Tyr Leu Thr Pro Gln
1185                1190                1195                1200

Gly Gly Ala Ala Pro Gln Pro His Pro Pro Pro Ala Phe Ser Pro Ala
                1205                1210                1215

Phe Asp Asn Leu Tyr Tyr Trp Asp Gln Asp Pro Pro Glu Arg Gly Ala
            1220                1225                1230

Pro Pro Ser Thr Phe Lys Gly Thr Pro Thr Ala Glu Asn Pro Glu Tyr
        1235                1240                1245

Leu Gly Leu Asp Val Pro Val
    1250                1255
```

We claim:

1. A method of screening for the presence of a malignancy in a warm-blooded animal, wherein a HER-2/neu oncogene is associated with the malignancy, comprising the steps of:

(a) contacting a body fluid, suspected of containing antibodies specific for HER-2/neu protein, with HER-2/neu protein;

(b) incubating the body fluid under conditions and for a time sufficient to allow immunocomplexes to form; and

(c) detecting the presence or absence of immunocomplexes formed between the HER-2/neu protein and antibodies in the body fluid specific for the HER-2/neu protein, thereby determining the presence or absence of the malignancy.

2. The method of claim 1 wherein a HER-2/neu oncogene is associated with a malignancy selected from the group consisting of breast, ovarian, colon, lung and prostate cancer.

3. The method of claim 1 wherein a reporter group is bound to a second antibody capable of binding to the antibodies, and wherein the step of detecting comprises (a) removing substantially any unbound antibody, (b) adding the second antibody, (c) removing substantially any unbound second antibody, and (d) detecting the presence or absence of the reporter group.

4. The method of claim 3 wherein the second antibody is an anti-human antibody.

5. The method of claim 3 wherein the reporter group is selected from the group consisting of radioisotopes, fluorophores, enzymes, luminescers, and dye particles.

6. The method of claim 1 wherein a reporter group is bound to a molecule capable of binding to the immunocomplexes, and wherein the step of detecting comprises (a) adding the molecule, (b) removing substantially any unbound molecule, and (c) detecting the presence or absence of the reporter group.

7. The method of claim 6 wherein the molecule capable of binding to the immunocomplexes is protein A.

8. The method of claim 6 wherein the reporter group is selected from the group consisting of radioisotopes, fluorophores, enzymes, luminascars, and dye particles.

9. The method of claim 1 wherein a reporter group is bound to the HER-2/neu protein, and wherein the step of detecting comprises removing substantially any unbound HER-2/neu protein and thereafter detecting the presence or absence of the reporter group.

10. The method of claim 9 wherein the reporter group is selected from the group consisting of radioisotopes, fluorophores, enzymes, luminascars, and dye particles.

11. A method for detecting changes in the level of antibodies specific for HER-2/neu protein in a human, comprising the steps of:

(a) contacting a first body fluid sample with HER-2/neu protein;

(b) incubating the sample under conditions and for a time sufficient to allow immunocomplexes to form;

(c) detecting immunocomplexes formed between the HER-2/neu protein and antibodies in the sample that are specific for the HER-2/neu protein;

(d) repeating steps (a), (b) and (c) on a second body fluid sample taken from the same individual at a time subsequent to the first sample; and

(e) comparing the number of immunocomplexes detected in the first and second body fluid samples, thereby determining whether a change in the level of antibody specific for HER-2/neu protein has occurred.

12. The method of claim **11** wherein a HER-2/neu protein is associated with a malignancy selected from the group consisting of breast, ovarian, colon, lung and prostate cancer.

13. The method of claim **11** wherein a reporter group is bound to a second antibody capable of binding to the antibodies, and wherein the step of detecting comprises (a) removing substantially any unbound antibody, (b) adding the second antibody, (c) removing substantially any unbound second antibody, and (d) detecting the presence or absence of the reporter group.

14. The method of claim **13** wherein the second antibody is an anti-human antibody.

15. The method of claim **13** wherein the reporter group is selected from the group consisting of radioisotopes, fluorophores, enzymes, luminescers, and dye particles.

16. The method of claim **11** wherein a reporter group is bound to a molecule capable of binding to the immunocomplexes, and wherein the step of detecting comprises (a) adding the molecule, (b) removing substantially any unbound molecule, and (c) detecting the presence or absence of the reporter group.

17. The method of claim **16** wherein the molecule capable of binding to the immunocomplexes is protein A.

18. The method of claim **16** wherein the reporter group is selected from the group consisting of radioisotopes, fluorophores, enzymes, luminescers, and dye particles.

19. The method of claim **11** wherein a reporter group is bound to the HER-2/neu protein, and wherein the step of detecting comprises removing substantially any unbound HER-2/neu protein and thereafter detecting the presence or absence of the reporter group.

20. The method of claim **19** wherein the reporter group is selected from the group consisting of radioisotopes, fluorophores, enzymes, luminescers, and dye particles.

* * * * *

US005728377A

United States Patent [19]

Sarris et al.

[11] Patent Number: 5,728,377

[45] Date of Patent: Mar. 17, 1998

[54] **METHODS AND COMPOSITIONS INCORPORATING IP-10**

[75] Inventors: **Andreas H. Sarris**, Houston, Tex.; **Hal E. Broxmeyer**, Indianapolis, Ind.; **Jeff V. Ravetch**, New York, N.Y.

[73] Assignees: **Board of Regents, The University of Texas System**, Austin, Tex.; **Indiana University Foundation**, Bloomington, Ind.; **Sloan-Kettering Institute for Cancer Research**, New York, N.Y.

[21] Appl. No.: **94,851**

[22] Filed: **Jul. 20, 1993**

[51] **Int. Cl.**[6] **A61K 45/05**

[52] **U.S. Cl.** **424/85.1**; 435/69.5; 530/351

[58] **Field of Search** 424/85.1; 435/69.5; 530/351; 935/13

[56] **References Cited**

FOREIGN PATENT DOCUMENTS

WO 93/13206 7/1993 WIPO .

OTHER PUBLICATIONS

Chen et al., "Carbohydrate Variant of the Recombinant β-Subunit of Human Choriogonadotropin Expressed in Baculovirus Expression System," *The Journal of Biological Chemistry*, 266(7):4081–4087, 1991.

Chen and Bahl, "Recombinant Carbohydrate Variant of Human Choriogonadotropin β-Subunit (hCGβ) Descarboxyl Terminus (115–145)," *The Journal of Biological Chemistry*, 266(10):6246–6251, 1991.

Chiou and Wu, "Expression of Human Granulocyte–Macrophage Colony–Stimulating Factor Gene in Insect Cells by a Baculovirus Vector," *FEBS Letters*, 259(2):249–253, 1990.

Chua and Blomberg, "Immunochemical Studies of Thylakoid Membrane Polypeptides for Spinach and *Chlamydomonas reinhardtii*," *The Journal of Biological Chemistry*, 254:215–223, 1979.

Davidson and Castellino, "Asparagine–Linked Oligosaccharide Processing in Lepidopteran Insect Cells. Temporal Dependence of the Nature of the Oligosaccharides Assembled on Asparagine–289 of Recombinant Human Plasminogen Produced in Baculovirus Vector Infected *Spodoptera frugiperda* (IPLB–SF–21AE) Cells," *Biochemistry*, 30:6165–6174, 1991.

Dimitriadis, "Effect of Detergents on Antibody–Antigen Interaction," *Analytical Biochemistry*, 98:445–451, 1979.

Gillespie et al., "Expression of Biologically Active Human Antithrombin III by Recombinant Baculovirus in *Spodoptera frugiperda* Cells," *The Journal of Biological Chemistry*, 266(6):3995–4001, 1991.

Ingley et al., "Production and Purfication of Recombinant Human Interleukin–5 from Yeast and Baculovirus Expression Systems," *Eur. J. Biochem.*, 196:623–629, 1991.

Jarvis and Summers, "Glycosylation and Secretion of Human Tissue Plasminogen Activator in Recombinant Baculovirus–Infected Insect Cells," *Molecular and Cellular Biology*, 9(1):214–223, 1989.

Lebacq–Verheyden et al., "Posttranslational Processing of Endogenous and of Baculovirus–Expressed Human Gastrin– Releasing Peptide Precursor," *Molecular and Cellular Biology*, 8(9):3129–3135, 1988.

Luckow and Summers, "High Level Expression of Nonfused Foreign Genes with *Autographa californica* Nuclear Polyhedrosis Virus Expression Vectors," *Virology*, 170:31–39, 1989.

Luster et al., "γ–Interferon Transcriptionally Regulates an Early–Response Gene Containing Homology to Platelet Proteins," *Nature*, 315(6021):672–676, 1985.

Miyajima et al., "Use of the Silkworm, *Bombyx mori*, and an Insect Baculovirus Vector for High–Level Expression and Secretion of Biologically Active Mouse Interleukin–3," *Gene*, 58:273–281, 1987.

Nakhai et al., "The α Subunith of Human Chorionic Gonadotropin Hormone Synthesized in Insect Cells using a Baculovirus Vector is Biologically Active," *FEBS Letters*, 283(1):104–108, 1991.

Quelle et al., "High–Level Expression and Purification of a Recombinant Human Erythropoietin Produced Using a Baculovirus Vector," *Blood*, 74(2):652–657, 1989.

Rodewald et al., "Production of Murine Interleukin–4 and Interleukin–5 by Recombinant Baculovirus," *Journal of Immunological Methods*, 132:221–226, 1990.

Sarris et al., "Recombinant Human IP–10: Expression, Biochemical Characterization, Purification and Inhibition of Early Human Hematopoietic Progenitors," *ISEH*, 1993, Abstract only.

Smith et al., "Production of Human Beta Interferon in Insect Cells Infected with a Baculovirus Expression Vector," *Molecular and Cellular Biology*, 3(12):2156–2165, 1983.

Smith et al., "Modification and Secretion of Human Interleukin 2 Produced in Insect Cells by a Baculovirus Expression Vector," *Proc. Natl. Acad. Sci. USA*, 82:8404–8408, 1985.

Steiner et al., "Human Tissue–Type Plasminogen Activator Synthesized by Using a Baculovirus Vector in Insect Cells Compared with Human Plasminogen Activator Produced in Mouse Cells," *Gene*, 73:449–457, 1988.

Whitefleet–Smith et al., "Expression of Human Plasminogen cDNA in a Baculovirus Vector–Infected Insect Cell System," Archives of Biochemistry and Biophysics, 271(2):390–399, 1989.

(List continued on next page.)

Primary Examiner—Toni R. Scheiner

Attorney, Agent, or Firm—Arnold, White & Durkee

[57] **ABSTRACT**

The present invention discloses the correct processing of IP-10, a myelosuppressive protein produced by certain cells such as keratinocytes, monocytes and human endothelial cells upon stimulation by γ-interferon. Also disclosed is a method of treating human cancer patients by applying IP-10 in a pharmaceutical composition in conjunction with certain antineoplastic agents.

15 Claims, 6 Drawing Sheets

OTHER PUBLICATIONS

Wojchowski et al., "Active Human Erythropoietin Expressed in Insect Cells Using a Baculovirus Vector: A Role for N-Linked Oligosaccharide," *Biochimica et Biophysica Acta*, 910:224-243, 1987.

Dewald et al., "IP-10, a γ-Interferon-inducible Protein Related to Interleukin-8, Lacks Neutrophil Activating Properties," *Immunology Letters*, 32:81-84, 1992.

Luster and Leder, "IP-10, a -C-X-C Chemokine, Elicits a Potent Thymus-dependent Antitumor Response In Vivo," *J. Exp. Med.*, 178:1057-1065, 1993.

Narumi et al., "Tissue-specific Expression of Murine IP-10 mRNA Following Systemic Treatment with Interferon γ," *Journal of Leukocyte Biology*, 52(1):27-33, 1992.

Ohmori and Hamilton, "A Macrophage LPS-Inducible Early Gene Encodes the Murine Homologue of IP-10," *Biochemical and Biophysical Research Communications*, 168(3):1261-1267, 1990.

Smoller and Krueger, "Detection of Cytokine-induced Protein γ-Immune Protein-10 (γ-IP10) in Atypical Melanocytic Proliferations," 25(4):627-631, 1991.

Broxmeyer, et al "Synergistic Suppressive Interactions of Human Chemokines on Human Myeloid Progenitor Cell Proliferation", Molecular Biology of Hematopoiesis 8th Symposium, Basel, Switzerland, Jul. 9-13, 1993, Abstract only.

Broxmeyer et al J of Immun. 150(8): 3448-4458 (Apr. 15, 1993).

Luster et al J of Exp Med. 166: 1084-1097, 1987.

Oppenheim *The Chemokines*, 1993, pp. 183-186.

Luster et al J of Ex Med 166: 1098-1108, 1987.

Luster et al, Keystone Symposia Jan./Feb. 1993, p. 121.

Harlow et al In *Antibiotics, A Lab. Manual*, Cold Spring Harbor Lab. 1988, pp. 285 and 287.

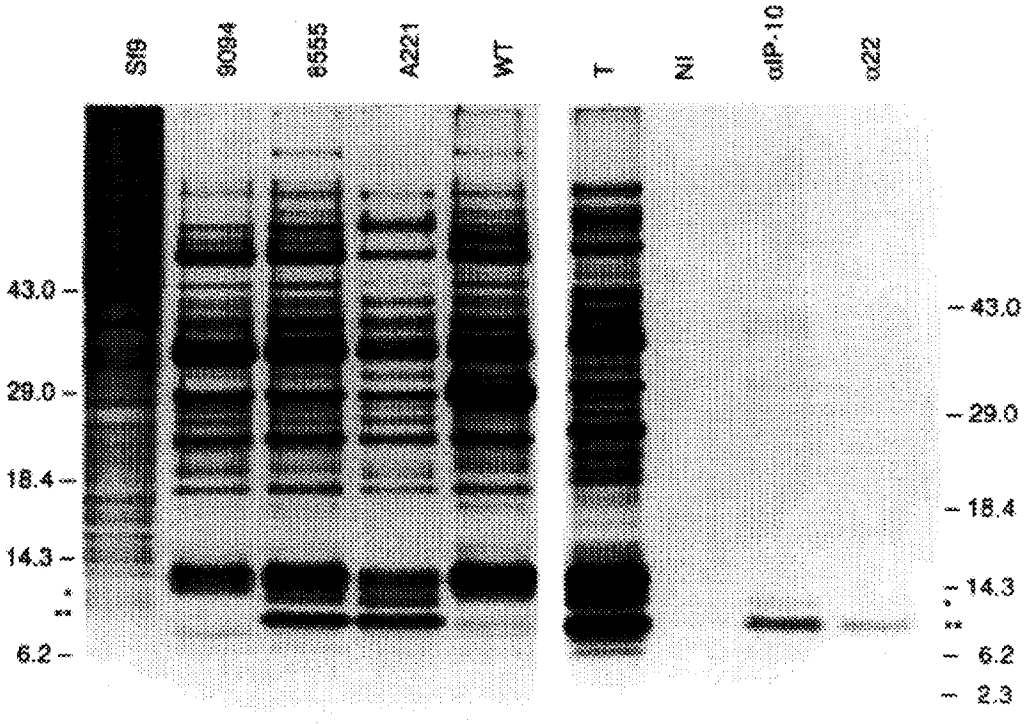


FIG. 1A FIG. 1B

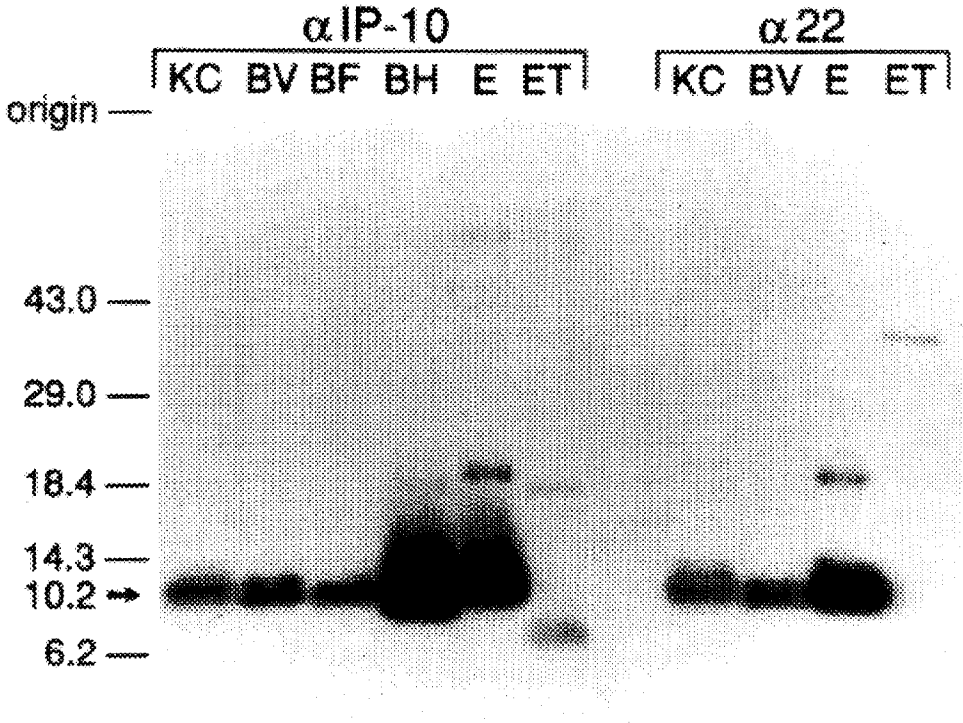

αIP-10
KC BV BF BH E ET
αIP-22
KC BV E ET
origin
43.0
29.0
18.4
14.3
10.2
6.2

FIG. 2A FIG. 2B

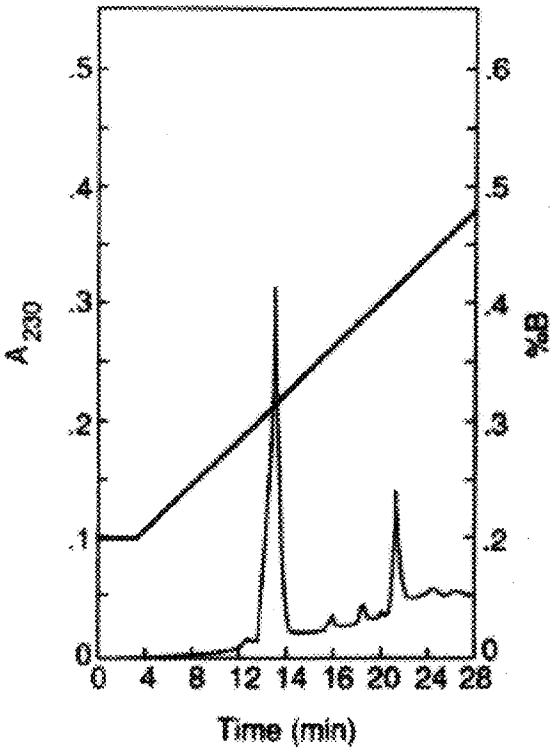

A_{280}
%B
0
.1
.2
.3
.4
.5
.6
0 4 8 12 14 16 20 24 28
Time (min)

FIG. 3A

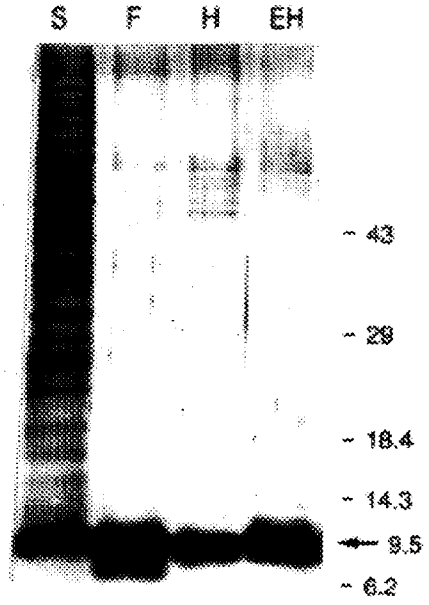

S
F
H
EH
~ 43
~ 29
~ 18.4
~ 14.3
9.5
~ 6.2

FIG. 3B

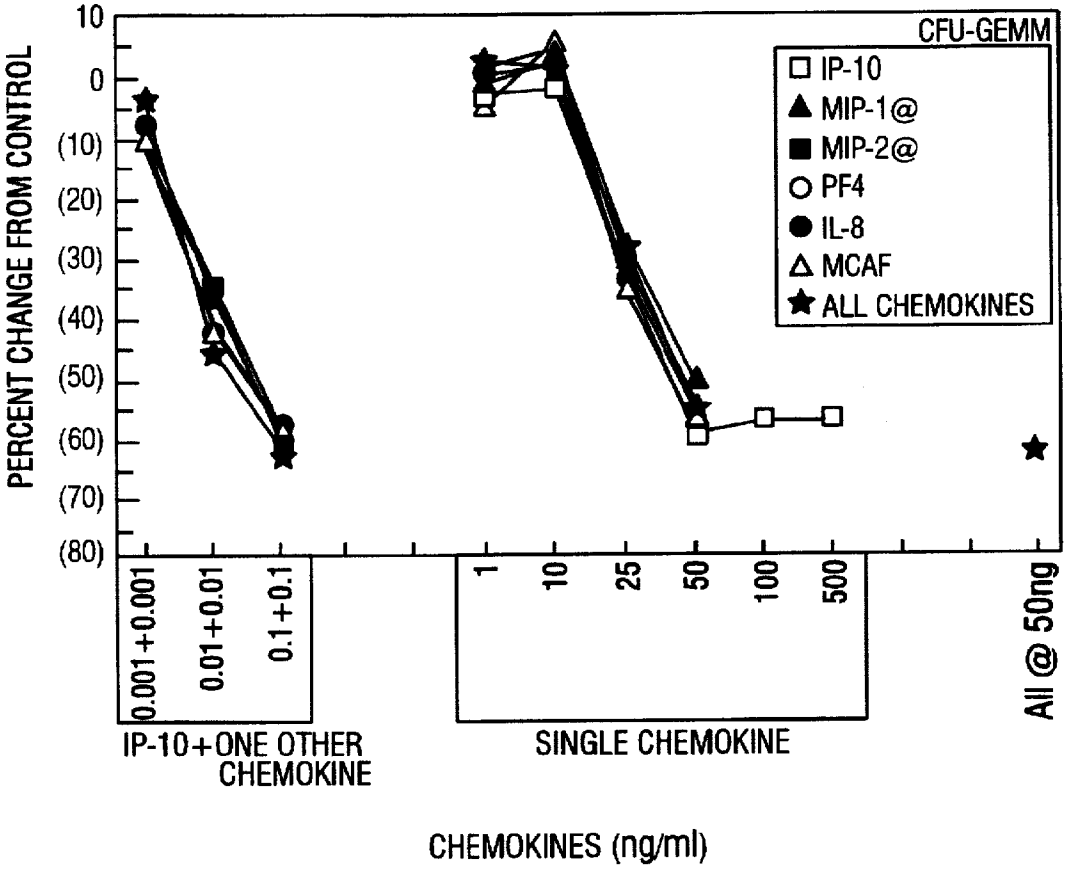

CFU-GEMM
IP-10
MIP-1@
MIP-2@
PF4
IL-8
MCAF
ALL CHEMOKINES
PERCENT CHANGE FROM CONTROL
10
0
(10)
(20)
(30)
(40)
(50)
(60)
(70)
(80)
0.001+0.001
0.01+0.01
0.1+0.1
1
10
25
50
100
500
All @ 50ng
IP-10+ONE OTHER CHEMOKINE
SINGLE CHEMOKINE
CHEMOKINES (ng/ml)

FIG. 4A

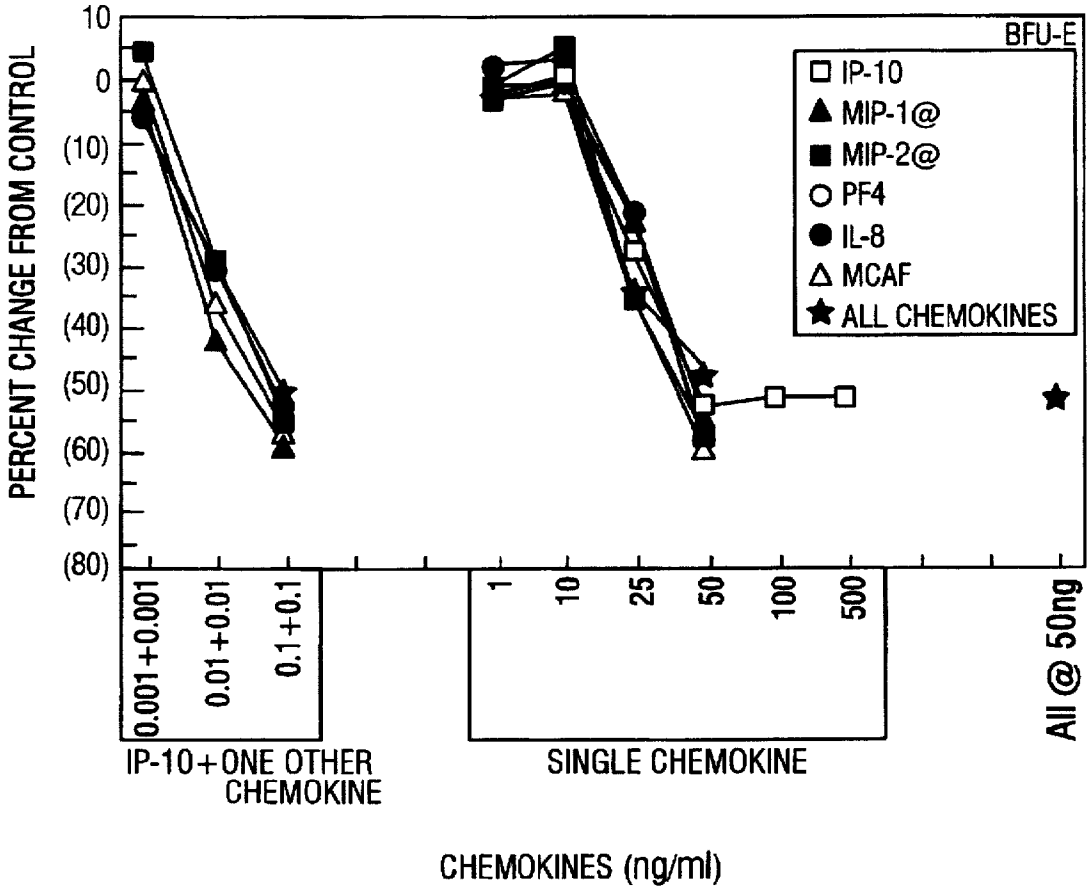

BFU-E
IP-10
MIP-1@
MIP-2@
PF4
IL-8
MCAF
ALL CHEMOKINES
PERCENT CHANGE FROM CONTROL
10
0
(10)
(20)
(30)
(40)
(50)
(60)
(70)
(80)
0.001+0.001
0.01+0.01
0.1+0.1
1
10
25
50
100
500
All @ 50ng
IP-10+ONE OTHER CHEMOKINE
SINGLE CHEMOKINE
CHEMOKINES (ng/ml)

FIG. 4B

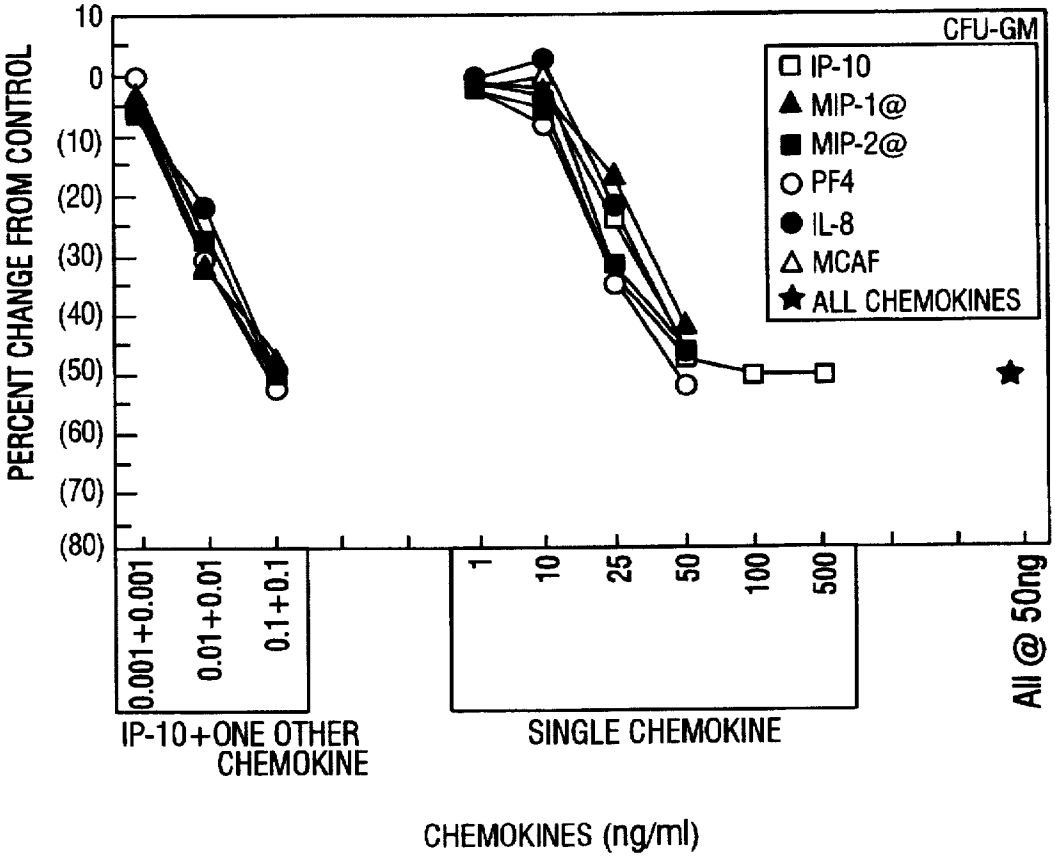

CFU-GM
IP-10
MIP-1@
MIP-2@
PF4
IL-8
MCAF
ALL CHEMOKINES
PERCENT CHANGE FROM CONTROL
10
0
(10)
(20)
(30)
(40)
(50)
(60)
(70)
(80)
0.001+0.001
0.01+0.01
0.1+0.1
1
10
25
50
100
500
All @ 50ng
IP-10+ONE OTHER CHEMOKINE
SINGLE CHEMOKINE
CHEMOKINES (ng/ml)

FIG. 4C

METHODS AND COMPOSITIONS INCORPORATING IP-10

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the field of the myelosuppressive action of a family of proteins that are secreted by certain types of cells in response to γ-interferon (γ-IFN), and more particularly to the myelosuppressive action of that protein known as IP-10, which is secreted by human keratinocytes, monocytes and endothelial cells.

2. Description of the Related Art

The cellular immune response is characterized by the complex interaction of many different cells responding to multiple extracellular signals. Activation, proliferation, and directed migration of both local and blood-borne cells is partly regulated by soluble mediators released by cells, referred to collectively as cytokines. One family of these proteins includes the interferons, a group of proteins first identified by their ability to induce cellular resistance to infection by many viruses. γ-IFN, a glycoprotein secreted from activated T cells, has potent immunomodulatory activities and is an important activator of the cellular immune response. In addition to the antiviral properties it shares with the α- and β- interferons, γ-IFN also activates macrophages and stimulates B cells.

IP-10 is a protein secreted by keratinocytes, monocytes and human endothelial cells after stimulation by recombinant γ-IFN. The gene encoding IP-10 has previously been cloned (Luster et al., 1985, Luster et al., 1987). DNA sequence analysis has demonstrated that IP-10 belongs to the intercrine cytokine or chemokine family of proteins (Wolpe et al., 1989; Oppenheim et al., 1991), so named for their chemotactic activity towards neutrophils, monocytes, T cells, basophils and fibroblasts. Some chemokines inhibit early subsets of bone marrow progenitors (Graham et al., 1990; Broxmeyer et al., 1990; Broxmeyer et al., 1991; Dunlop et al., 1992; Lord et al., 1992; Maze et al., 1992; Broxmeyer et al., 1993). This family is divided into two subgroups based on the arrangement of the first two of four conserved cysteines: the α-subfamily with the C—X—C motif, which is located on human chromosome 4 (q12–21) and includes GRO-α, β-thromboglobulin, MIP-2α, MIP-2β, IL-8, NAP-2, and IP-10; and the β-subfamily with the C—C motif, which is located on human chromosome 17 (q11–32) and includes MIP-1α, MIP-1β, MCAF, and RANTES (Wolpe et al., 1989; Oppenheim et al., 1991).

Even though recombinant IP-10 has been expressed and the purified recombinant protein has been available, no practical usefulness for IP-10 has been reported. On the contrary, previous attempts to demonstrate a biological function for IP-10 have been unsuccessful (Dewald et al., 1992). Furthermore, the final processed form of IP-10 has not been known, rendering uncertain the preparation of appropriate recombinant vectors and recombinant protein for further study of the biological function of this protein. Therefore, since so little is known about the biological function of IP-10, there is a continuing need in the art to characterize, understand and ultimately exploit the activity of this protein.

SUMMARY OF THE INVENTION

The present invention involves, in a general and overall sense, the discovery by the inventors that IP-10 directly inhibits the growth of early bone marrow or hematopoietic progenitors and thus can be employed in a variety of embodiments, including clinical compositions and methods. Most importantly, in that the present inventors herein demonstrate that IP-10 can inhibit proliferation of early human bone marrow myeloid progenitors, it is envisioned that IP-10 can be employed to protect such cells from the adverse effects of agents that selectively exert a cytotoxic effect upon normal, dividing bone marrow progenitors.

While many cancer cell types are characterized by uncontrolled growth characteristics, there have been few other cancer-specific biological or cellular targets for the development of directed chemotherapy. Accordingly, most traditional cancer therapies are very nonspecific in that they employ antineoplastic agents that indiscriminantly kill rapidly growing cells and cell types, whether cancerous or not. The most susceptible normal tissues to the ravaging effects of chemotherapy are thus rapidly growing cell types such as bone marrow progenitor cells and cells of the gastrointestinal tract. The present invention thus embodies distinct advantages as, through the administration of pharmaceutical compositions of IP-10, it is proposed that one can induce a non-dividing state in hematopoietic progenitor cells, protecting them from the anticellular effects of cell cycle specific agents, such as hydroxyurea, cytosine arabinoside, methotrexate and vincristine.

The inventors also contemplate that IP-10 may have utility as a direct inhibitor of tumor cell growth. While the effect of IP-10 upon leukemic cells is presently unknown, it may exert a direct inhibitory effect upon sarcomas such as Kaposi's sarcoma, by virtue of its similarity to Platelet Factor 4, which is active against Kaposi's sarcoma in AIDS patients (Kahn et al., 1993). In connection with the treatment of Kaposi's sarcoma, it is contemplated that IP-10 may be particularly useful because as presently employed, it does not promote HIV replication. Similarly, with respect to the treatment of leukemia patients, IP-10 may ultimately demonstrate strong inhibitory effects against leukemic stem cells that are known to depend on steel factor. It is proposed that in these patients IP-10 can be employed directly as an anti-leukemic agent. In patients wherein IP-10 does not inhibit leukemic cells, it can be employed in the treatment of leukemia as an adjunct to traditional chemotherapy as discussed above, because it will protect normal bone marrow stem cells, but not leukemic cells.

Accordingly, it can be appreciated that the present invention involves, in certain more general embodiments, methods for inhibiting the growth of early hematopoietic progenitor cells. Such methods include contacting a population of cells that include hematopoietic progenitor cells with an effective amount of IP-10. Effective amounts of IP-10 will be those quantities of IP-10 which are effective to inhibit early hematopoietic progenitor cell growth, as exemplified herein. While it is generally believed that IP-10 will prove useful in inhibiting all such early progenitor cells, the cells typically observed to be inhibited by recombinant IP-10 ("rIP-10") include early subsets of granulocyte-macrophage progenitor cells (CFU-GM), multipotential (CFU-GEMM) and erythroid (BFU-E) progenitors that depend on rSLF in addition to rGM-CSF or rEPO respectively.

Surprisingly, inactive concentrations of rIP-10 combined with inactive concentrations of rMIP-1α, rMIP-2α, PF4, rIL-8, or rMCAF have been found to result in synergistic inhibition of early progenitors. Therefore, further aspects of the present invention include compositions comprising IP-10, such as rIP-10, which may or may not have activity individually, in combination with any one or more of rMIP-1α, rMIP-2α, PF4, rIL-8, or rMCAF, at concentrations which may or may not have activity individually.

In preferred aspects, the invention concerns the generation and use of recombinant IP-10 which is produced through the application and use of recombinant DNA segments comprising the IP-10 gene (see Luster et al., 1985 and Luster et al., 1987). It is proposed that any method for recombinant production will be suitable for use in connection with the present invention. However, an aspect of the invention is the inventors' discovery of the correct structure of the active, naturally secreted form of IP-10, which is initially produced with a 21 amino acid leader sequence that is cleaved off during secretion, leaving the so-called f(22–98) (IP-10 fragment composed of amino acids 22–98) as the active, secreted form of the protein.

Thus, if one envisions employing a bacterial recombinant expression system such as *E. coli*, it will be appropriate to prepare a vector construct that will directly encode and thereby produce f(22–98) IP-10. This is because bacterial expression systems will be unable to remove that leader sequence from the finished protein. However, where a eukaryotic recombinant cell expression system such as an insect cell or a mammal or even a human cell is employed, one will not be required to prepare a truncated IP-10 expression segment in that the host system itself will remove the leader sequence of 21 amino acids (assuming that the cDNA encoding the full length, secreted form of IP-10 is employed).

Certain aspects of the present invention therefore concern methods for inhibiting the growth of hematopoietic progenitor cells which include preparing recombinant IP-10 (rIP-10) and contacting a composition containing such cells with an effective amount of rIP-10. rIP-10 may be prepared by any suitable process, such as, e.g., by preparing a recombinant host cell which includes a recombinant gene segment encoding IP-10, culturing the cell under conditions effective to allow the expression of the gene so that the recombinant protein is produced and collecting and purifying the IP-10 so produced.

It is understood that the recombinant IP-10 may be produced in a bacterial or prokaryotic cell, or in an insect, animal or human cell. Collecting the IP-10 may take the form of collecting media or 'conditioned media' surrounding the recombinant cells, or may include first lysing or otherwise breaking the cells and generally removing any cell debris. Various techniques are suitable for use in protein purification, virtually any of which may be employed to purify recombinant IP-10. These include, but are not limited to: precipitation, e.g., using salt, antibodies, ammonium sulphate, PEG, and the like, followed by centrifugation; chromatography steps such as ion exchange, gel filtration and reverse phase chromatography, any of which may also be used with FPLC and HPLC, and also hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis including SDS-PAGE and gel-elution; and combinations of such and other techniques known to those of skill in the art. The preferred method for purifying IP-10 disclosed herein involves immunoprecipitation and affinity chromatography and represents the best mode presently known by the inventors to prepare substantially purified IP-10.

The inventors contemplate that the purified IP-10 so produced may be comprised in a pharmaceutical composition and that the said pharmaceutical composition may be administered to a human cancer patient. This administration of IP-10 may be followed by the administration of an anti-neoplastic agent which is toxic to bone marrow cells and which is administered in a therapeutically effective amount. Therefore, a certain embodiment of the present invention comprises an improved method for chemotherapy of a cancer patient employing an antineoplastic agent having bone marrow toxicity, wherein the improvement comprises treating said patient with an early hematopoietic progenitor cell inhibitory amount of IP-10.

Pharmaceutical compositions in accordance with the present invention may therefore include purified IP-10, and preferably rIP-10; or rIP-10 in combination with another beneficial agent, such as a cytokine; or rIP-10 in combination with an anti-neoplastic agent. Anti-neoplastic agents include, but are not limited to alkylating agents; antimetabolites, such as purine and pyrimidine analogs and vinca alkaloids; natural products including antibiotics and miscellaneous agents used in the treatment of various neoplastic diseases. Specific examples of such compounds include doxorubicin, daunomycin, methotrexate, vinblastine, hydroxyurea and cytosine arabinoside.

The improved method for inhibiting the growth of hematopoietic progenitor cells in a patient may comprise the steps of preparing a recombinant host cell comprising a recombinant gene segment encoding IP-10, culturing said cell under conditions effective to allow expression of said gene to produce IP-10; collecting and purifying the recombinant IP-10 so produced; rendering said recombinant IP-10 pharmacologically acceptable; and administering said pharmacologically acceptable IP-10 to said patient in an amount effective to inhibit hematopoietic progenitor cells.

In preferred embodiments, the rIP-10 is produced from a baculovirus expression vector in an insect host cell. The IP-10 may be produced from a recombinant gene segment which encodes f(22–98) or it may encode the entire 98 amino acid sequence of IP-10, or it may be naturally occurring IP-10, derived from stimulated keratinocytes, monocytes or endothelial cells, for example. The IP-10 may be purified and rendered pharmacologically acceptable and sterile as described herein, and as known to those of skill in the art, and administered to a human cancer patient to whom is also administered an agent, an antineoplastic agent for example, having bone marrow toxicity.

It is understood that the IP-10 which is used in the pharmaceutical composition may be prepared by a process that includes preparing a recombinant host cell comprising a recombinant gene segment encoding IP-10; culturing said cell under conditions effective to allow expression of said gene to produce IP-10; and collecting and purifying the IP-10 so produced. The composition will generally comprise from about 99 to about 99.99% IP-10, and more preferably from about 100 to about 200 μg/ml of IP-10.

The inventors' contemplate that the pharmaceutical composition may be prepared by a method comprising the steps of obtaining a composition that includes IP-10; purifying the IP-10 from said composition by removing one or more impurities therefrom; and rendering the purified composition pharmacologically acceptable to provide the pharmaceutical composition. The term "pharmaceutically acceptable" refers to molecular entities and compositions which do not produce an adverse, allergic or other untoward reaction when administered to a human. The preparation of suitable pharmacological compositions is routine to those of skill in the art, as exemplified by "Remington's Pharmaceutical Sciences" 15th Edition, incorporated herein by reference.

The composition that includes IP-10 may be obtained by stimulating keratinocytes, monocytes, endothelial cells or others with an amount of γ-interferon effective to promote the release of IP-10 by said cells, and collecting and partially purifying the IP-10 so released to provide the composition or

by preparing a recombinant host cell comprising a recombinant gene segment encoding IP-10; culturing said cell under conditions effective to allow expression of said gene to produce IP-10; and collecting and purifying the IP-10 so produced. The recombinant gene segment may encode f(22–98) IP-10 or it may encode full length IP-10. It is understood that the IP-10 described in this paragraph may be produced from a baculovirus expression vector or by any other means.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A and 1B. SDS-PAGE analysis of protein synthesis by various baculovirus vectors.

FIG. 1A. Protein synthesis by wild type and recombinant baculoviruses. Cells were pulsed with [^{35}S]-methionine from 60 to 66 hours post-infection, washed, boiled in sample buffer and analyzed by SDS-PAGE. The top of each lane designates the infecting baculovirus. WT: wild type baculovirus. Sf9: un-infected cells.

FIG. 1B. Immunoprecipitations of cell-associated rIP-10. Sf9 cells were infected with A221, pulsed with [^{35}S]-methionine from 60 to 66 hours post infection, boiled in SDS (T), and immunoprecipitates of an equal number of cells by non-immune serum (NI), anti-IP-10, or anti-22 were analyzed with SDS-PAGE followed by fluorography, as designated at the top of the corresponding lanes. * designates the 11.9 kDa form, and ** the 9.9 kDa form of rIP-10.

FIGS. 2A and 2B. Western blot analysis of natural and rIP-10. Keratinocyte IP-10 (KC), supernatants from A221-infected Sf9 cells (BV), and representative fractions from Sepharose-S FPLC (BF) or C4 reverse phase HPLC (BE) were analyzed by SDS-PAGE along with f(22–98) (E) or f(22–77) (ET).

FIG. 2A is a duplicate membrane stained with anti-IP-10.

FIG. 2B is a duplicate membrane stained with anti-22.

FIGS. 3A and 3B. Purification of rIP-10 from baculovirus-infected cells.

FIG. 3A. Reverse-phase HPLC of rIP-10 purified from Sf9 cells infected with A221. A_{230} is the optical density of the eluate at 230 nm and % B is the gradient of propanol-acetonitrile-TFA.

FIG. 3B. Analysis of the purification of rIP-10 by SDS-PAGE. S, supernatants of A221-infected Sf9 cells 6 days after infection; F, representative fractions of FPLC on Sepharose-S; H, peak fraction of reverse-phase HPLC shown in 3A; and E, purified f(22–98) from *E. coli.*

FIGS. 4A, 4B and 4C. Influence of rIP-10 on colony formation. CFU-GM were plated in the presence of rGM-CSF (100 U/ml) and rSLF (50 ng/ml). CFU-GEMM and BFU-E were plated in the presence of rEPO (1 U/ml) and rSLF (50 ng/ml). Results are given as the mean±SEM and reflect 3 separate experiments with rIP-10, and 2–3 experiments for the other chemokines. Percent changes from control designate suppression and were based on control colony numbers for CFU-GM (59±2 to 106±7), BFU-E (58±4 to 105±2), and CFU-GEMM (37±3 to 68±1). Most symbols in the graphs are smaller than the SEM, which was always ≦12% of the mean percent change.

FIG. 4A. Effect of rIP-10 on CFU-GEMM.

FIG. 4B. Effect of rIP-10 on BFU-E.

FIG. 4C. Effect of rIP-10 on CFU-GM.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

A particular embodiment of the present invention is the use of a processed, recombinant protein in a pharmaceutical preparation in conjunction with chemotherapy of cancer patients. IP-10, a member of the family of small secreted proteins called intercrine cytokines or chemokines, is secreted by γ-IFN stimulated T cells, monocytes, endothelial cells and keratinocytes. The biological properties of IP-10 can now be explored due to the cloning of the gene and overexpression in baculovirus and in bacterial protein expression systems. A 9.9 kDa protein was secreted by insect cells infected with a baculovirus vector containing the IP-10 gene. The said 9.9 kDa protein co-migrated with keratinocyte IP-10 and with f(22–98), a bacterial recombinant fragment lacking the signal sequence, but containing all other residues of IP-10 on SDS-PAGE. All three polypeptides reacted with antibodies recognizing residues 10–98 (anti-IP-10) and 77–98 (anti-22) of IP-10, demonstrating that IP-10 is secreted by keratinocytes and infected insect cells after removal of the signal sequence. An important discovery of the present invention is the fact that the IP-10 protein does not undergo proteolysis of the carboxy-terminal end as was previously believed. It is the inventors' belief, in fact, that the deletion of the 21 carboxy-terminal residues of IP-10 results in an inactive protein, in direct contradiction of the previous model (Luster and Ravetch, 1987).

Purified rIP-10 suppresses in vitro colony formation by early human bone marrow progenitor cells which need r-Steel Factor (rSLF) and r-granulocyte-macrophage CSF (rGM-CSF) or rSLF and r-erythropoietin (rEPO). The inhibition is dose-dependent, is complete at concentrations ≧50 ng/ml, is prevented by preincubation of rIP-10 with anti-IP-10, but not by anti-22, and is also seen with highly purified CD-34^{+++} cells, suggesting direct effect by rIP-10 on the progenitors. Combinations of rIP-10 and other chemokines at inactive concentrations inhibited colony formation in a synergistic manner. rIP-10 did not affect colony formation in the absence of any growth factors or in the presence of rEPO or rGM-CSF but in absence of rSLF. The effects of IP-10 may be relevant to normal marrow function and might be harnessed to protect human hematopoietic progenitors from the cytotoxic effects of chemotherapy.

Abreviations

The following abbreviations are used throughout the present disclosure:

SLF, steel factor; EPO, erythropoietin; CFU-GM, CFU-granulocyte-macrophage; CFU-GEMM, CFU-granulocyte-erythroid-macrophage-megakaryocytic; BFU-E erythroid burst forming unit; PF4, platelet factor-4; FPLC, fast performance liquid chromatography; MIP, macrophage inflammatory protein; NAP, neutrophil activating peptide; MCAF, macrophage chemotactic and activating factor; IL, interleukin.

Cloning and Protein Purification

A technique often employed by those skilled in the art of protein production today is to obtain a so-called "recombinant" version of the protein, to express it in a recombinant cell and to obtain the protein from such cells. These techniques are based upon the "cloning" of a DNA molecule encoding the protein from a DNA library, i.e., on obtaining a specific DNA molecule distinct from other portions of DNA. This can be achieved by, for example, cloning a cDNA molecule, or cloning a genomic-like DNA molecule.

The first step in such cloning procedures is the screening of an appropriate DNA library, such as, in the present case, a human histiocytic lymphoma cell line. The screening procedure may be an expression screening protocol employing antibodies directed against the protein, or activity assays. Alternatively, screening may be based on the hybridization of oligonucleotide probes, designed from a consideration of

portions of the amino acid sequence of the protein, or from the DNA sequences of genes encoding related proteins. After identifying an appropriate DNA molecule, it may be inserted into any one of the many vectors currently known in the art and transferred to a prokaryotic or eukaryotic host cell where it will direct the expression and production of the so-called recombinant version of the protein.

Alternatively, a cDNA library may be constructed from poly $(A)^+$ RNA isolated from cells such as the U937 cell line. This RNA may then be translated in a cell free system such as the rabbit reticulocyte system. The translation products may then be analyzed by SDS-PAGE, for example. It is understood also, that a subtraction hybridization system may be employed to discover mRNA that is only synthesized in response to certain stimuli, γ-IFN, for example.

It will be understood that recombinant IP-10 may differ from naturally-produced IP-10 in certain ways. In particular, the degree of post-translational modifications, such as, for example, glycosylation and phosphorylation may be different between the recombinant IP-10 and the IP-10 purified from a natural source, such as keratinocytes.

Protein Purification

Further aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of a protein. The term "purified protein" as used herein, is intended to refer to a protein composition, isolatable from total serum protein, wherein the protein is purified to any degree relative to its naturally-obtainable state, i.e., in this case, relative to its purity within a serum extract. A purified protein therefore also refers to a protein, free from the environment in which it may naturally occur.

Various methods for quantifying the degree of purification of the protein will be known to those of skill in the art in light of the present disclosure. These include, for example, assessing the number of polypeptides within a fraction by SDS/PAGE analysis.

The actual units used to represent the amount of inhibitory activity will, of course, be dependent upon the particular assay technique chosen to follow the purification. As discussed below, the present inventors prefer to use an assay based upon the suppression of colony growth of CFU-GM in the presence of rGM-CSF and rSLF; or the inhibition of BFU-E or CFU-GEMM in the presence of rSLF and rEPO.

Generally, "purified" will refer to a protein composition which has been subjected to fractionation to remove various non-protein components or other irrelevant proteins. Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques.

The preferred purification method disclosed hereinbelow contains several steps and represents the best mode presently known by the inventors to prepare a substantially purified IP-10. This method is currently preferred as it results in the substantial purification of the protein, as assessed by colony suppressing activity. This preferred mode of protein purification involves the execution of certain purification steps in the order described hereinbelow. However, as is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified IP-10.

Keratinocyte expression

Primary human keratinocytes were grown in serum-free media (Krueger, 1990), and induced with 500 U/ml rIFN-γ. The supernatant was collected after 24 hours, and concentrated 10-fold with trichloroacetic acid precipitation before western blotting. Keratinocytes were labelled with ^{35}S-methionine and IP-10 immunoprecipitated from their supernatants as previously described (Luster, 1987; Krueger, 1990).

Recombinant Host Cells and Vectors

Prokaryotic hosts are preferred for expression of the unprocessed IP-10 protein, or for any defined fragment of IP-10. Some examples of prokaryotic hosts are *E. coli* BL21 (DE3) which is particularly useful. Others are *E. coli* LE392, *E. coli* B, *E. coli* RR1, *E. coli* X 1776 (ATCC No. 31537) as well as *E. coli* W3110 (F-, lambda-, prototrophic, ATCC No. 273325), bacilli such as *Bacillus subtilis,* or other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcescens,* and various Pseudomonas species may be used.

More particularly, rIP-10 has been produced using the T7 polymerase bacterial expression system (provided by F W Studier, Brookhaven National Laboratories, New York, Studier, 1990). The polymerase chain reaction and appropriate oligonucleotide primers (Saiki, 1988) were used to amplify fragments of IP-10 cDNA with NcoI ends for cloning in the protein expression vector peT-3d. Oligonucleotides were synthesized on a model 380B synthesizer from Applied Biosystems (Foster City, Calif.) using phosphoramidite chemistry. The primers used were OL1075 (GGATCCATGGTACCTCTCTCTAGAACC; seq id no:1) as the 5' and OL 1076 (GGATCCATGGTTAAGGAGATCTTTTAGA; seq id no:2) as the 3' primer. A cDNA coding for f(22–98), a fragment extending from Valine 22 to Proline 98 and lacking the signal sequence of IP-10 was amplified. In order to approximate the previously reported processed form of IP-10 (Luster, 1987), OL1075 was used as the 5' primer and OL1077 (GGATCCATGGTTATGGATTCAGACATCTCTT; seq id no:3) as the 3' primer. This procedure amplified f(22–77), a cDNA coding for a fragment extending from Valine 22 to Proline 77, and lacking both the signal sequence and the last 21 residues of IP-10, but retaining the 4 internal cysteine residues. The PCR products were restriction digested with NcoI and were cloned into peT-3d, thus removing all residues of the ϕ10 protein (Studier, 1990), and expressing the IP-10 fragments as nonfusion proteins with an added methionine at the amino terminus. The IP-10 coding regions of the recombinant plasmids were sequenced (Sanger, 1977) to eliminate the possibility of PCR and cloning errors. Recombinant plasmids were transformed into lysogen BL21 (DE3) and transformants were induced with 0.4 mM IPTG (Studier, 1990). Recombinant f(22–98) was solubilized (Marston) and purified as described for neutrophil activating peptide (Lindley, 1988), using 40 mM Na Phosphate pH 7.2 during chromatography in S-Sepharose. Fractions with IP-10 were pooled and dialysed against PBS.

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures may also be used. *Saccharomyces cerevisiae,* or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in Saccharomyces, the plasmid YRp7, for example, is commonly used (Stinchcomb et al., 1979; Kingsman et al., 1979; Tschemper et al., 1980). This plasmid already contains the trpl gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, 1977). The

presence of the trpl lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., 1980) or other glycolytic enzymes (Hess et al., 1968; Holland et al., 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination. Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Any plasmid vector containing a yeast-compatible promoter, an origin of replication, and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (*Tissue Culture,* 1973). Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7, 293 and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

Of particular interest is the baculovirus system in which Sf9 insect cells infected with recombinant baculoviruses synthesize large amounts of recombinant proteins. Of particular importance is the ability of Sf9 cells to remove leader sequences, correctly fold proteins, and secrete them in the media (Webb and Summers, 1990; Summers and Smith, 1987). Many secreted proteins have been successfully produced in soluble, biologically active form from Sf9 cells infected by recombinant baculoviruses (Smith, 1985; Miyajima, 1987; Rodewald, 1990; Ingley, 1991; Smith, 1983; LeBacq-Verheyden, 1988; Whitefleet-Smith, 1989; Davidson, 1991; Steiner, 1988; Jarvis, 1989; Wojchowski, 1987; Quelle, 1989; Barnett, 1990; Chiou, 1990; Nkhai, 1991; Chen, 1991; Chen, 1991; Gillepsie, 1991).

DNA Segments

Important aspects of the present invention concern isolated DNA segments and recombinant vectors encoding the IP-10 protein, and the creation of recombinant host cells, through the application of DNA technology, which express IP-10.

As used herein, the term "DNA segment" is intended to refer to a DNA molecule which has been isolated free of total genomic DNA of a particular species. Therefore, a DNA segment encoding IP-10 is intended to refer to a DNA segment which contains such coding sequences, yet is isolated away from total genomic DNA of the human cell. Included within the term "DNA segment", are DNA segments which may be employed in the preparation of vectors, as well as the vectors themselves, including, for example, plasmids, cosmids, phage, viruses, and the like.

Recombinant vectors and isolated segments may variously include the IP-10 coding regions themselves, coding regions bearing selected alterations or modifications in the basic coding region or may encode larger polypeptides which nevertheless include sequences which will define the myelosuppressive activity.

Site-Specific Mutagenesis

Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functionally equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art as exemplified by publications (Adelman et al., 1983). As will be appreciated, the technique may employ a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage (Messing et al., 1981). These phage are readily commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis which eliminates the step of transferring the gene of interest from a plasmid to a phage.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector or melting apart the two strands of a double stranded vector which includes within its sequence a DNA sequence which encodes the IP-10 protein. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example by the method of Crea et al. (1978). This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected IP-10 encoding gene using site-directed mutagenesis is provided as a means of producing potentially useful protein species and is not meant to be limiting as there are other ways in which sequence variants of the protein may be obtained. For example, recombinant vectors encoding the desired IP-10 encoding gene may be treated with mutagenic agents to obtain sequence variants (see, e.g., a method described by Eichenlaub, 1979) for the mutagenesis of plasmid DNA using hydroxylamine.

Protein Modifications

As mentioned above, modification and changes may be made in the structure of IP-10 and still obtain a molecule having like or otherwise desirable characteristics. For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules or receptors. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence (or, of course, its underlying DNA coding sequence) and nevertheless obtain a protein with like, enhanced or even countervailing properties (e.g., antagonistic v. agonistic). It is thus contemplated by the inventors that various changes may be made in the sequence of IP-10 proteins or peptides (or underlying DNA) without appreciable loss of their biological utility or activity, and changes may also be made which enhance their biological utility or activity.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte & Doolittle, J. Mol. Biol., 157:105–132, 1982). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (−0.4); threonine (−0.7); serine (−0.8); tryptophan (−0.9); tyrosine (−1.3); proline (−1.6); histidine (−3.2); glutamate (−3.5); glutamine (−3.5); aspartate (−3.5); asparagine (−3.5); lysine (−3.9); and arginine (−4.5).

It is believed that the relative hydropathic character of the amino acid determines the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, antibodies, antigens, and the like. It is known in the art that an amino acid may be substituted by another amino acid having a similar hydropathic index and still obtain a biological functionally equivalent protein. In such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

Substitution of like amino acids can also be made on the basis of hydrophilicity, particularly where the biological functional equivalent protein or peptide thereby created is intended for use in immunological embodiments. U.S. Pat. No. 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, i.e. with a biological property of the protein.

As detailed in U.S. Pat. No. 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0±1); glutamate (+3.0±1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (−0.4); proline (−0.5±1); alanine (−0.5); histidine (−0.5); cysteine (−1.0); methionine (−1.3); valine (−1.5); leucine (−1.8); isoleucine (−1.8); tyrosine (−2.3); phenylalanine (−2.5); tryptophan (−3.4). It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine. For example, site specific mutagenesis of phenylalanine 56 of IP-10 to tyrosine may permit the generation of a biologically active molecule able to bind on the surface of early hematopoietic progenitors or appropriate cell lines. The tyrosine can be labeled with radioactive iodine without destroying the biological activity of the protein, and thus would allow the study and isolation of the cellular receptors of IP-10.

While discussion has focused on functionally equivalent polypeptides arising from amino acid changes, it will be appreciated that these changes may be effected by alteration of the encoding DNA; taking into consideration also that the genetic code is degenerate and that two or more codons may code for the same amino acid.

In addition, deletion mutations of IP-10 can be generated with the polymerase chain reaction, as is the case of f(22–77) which lacks the last 21 carboxy-terminal residues of IP-10 and is believed to be inactive. This discovery enhances the value of the correct definition of the natural form of IP-10 contained within the present disclosure.

Pharmaceutical Preparations

The novel pharmaceutical preparations of the present invention may be administered alone or in combination with pharmaceutically acceptable carriers, in either single or multiple doses. Suitable pharmaceutical carriers include inert solid diluents or fillers, sterile aqueous solution and various organic solvents. The pharmaceutical compositions formed by combining a protein of the present invention and the pharmaceutically acceptable carriers are then easily administered in a variety of dosage forms such as injectable solutions.

For parenteral administration, solutions of the protein in sesame or peanut oil, aqueous propylene glycol, or in sterile aqueous solution may be employed. Such aqueous solutions should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils.

The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. It is also contemplated that dilute acidic solutions may be preferred in order to maintain IP-10 in solution. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus ny additional desired ingredient from a previously sterile-filtered solution thereof.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

Suppression of Stem Cells During Chemotherapy

In a certain embodiment, the myelosuppressive activity of the pharmaceutical preparations discussed above may prove useful in the treatment of human cancers. For example, chemotherapeutic agents are often designed to have a selective effect on rapidly growing cells. Unfortunately, healthy bone marrow, hair follicle and intestinal epithelium are also affected by these agents. The present invention, by suppressing growth in at least some of these cells may protect them from the chemotherapy agents and thus allow for a more intense or longer period of chemotherapy treatment.

The preparations of the present invention may be used with a variety of chemotherapy agents. Some classes of agents include alkylating agents such as nitrogen mustards, ethylenimines and methylmelamines, alkyl sulfonates, nitrosoureas, triazenes and the like; antimetabolites such as folic acid analogs, pyrimidine analogs, in particular fluorouracil and cytosine arabinoside, and purine analogs and the like; natural products such as vinca alkaloids, epipodophyllotoxins, antibiotics, enzymes and biological response modifiers; and miscellaneous products such as platinum coordination complexes, anthracenedione, substituted urea such as hydroxyurea, methyl hydrazine derivatives, adrenocorticoid suppressants and the like. See for example, Goodman and Gilman's *The Pharmacological Basis of Therapeutics*, 8th edition, Pergamon Press, section XII, incorporated herein by reference.

The chemotherapy agents are administered orally, intravenously, intramuscularly, intrapleurally or intraperitoneally at doses based on the body weight and degree of disease progression of the patient, and may be given in one, two or even four daily administrations. Dosages range from about 0.5 mg/kg per day up to tens and even hundreds of mg/kg per day depending on the chemotherapy agent being administered and the patients weight and condition. The preparation of the present invention may be administered in conjunction with the chemotherapeutic agent. "In conjunction with" means that the pharmaceutical composition is administered either in a single composition or separately, either before, after or concurrently with the chemotherapy agent. Dosages of the myelosuppressive preparation will also depend on the body weight of the patient and on the amount of chemotherapy agent administered. Dosages will range from about 3 μg/kg per day to about 100 μg/kg per day, and may be administered intravenously, intramuscularly or intraperitoneally, for example. The exact dosage will be determined by the practitioner for each individual case.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE I

Cloning of IP-10

The cloning of IP-10 cDNA and the affinity purification of antibodies against residues 10–98 (anti-IP-10), and 77–98 (anti-22) of IP-10 have been described elsewhere (Luster et al., 1985, Luster et al., 1987). Below is a brief description of the procedures.

Cell Culture

Human endothelial cells were isolated from umbilical cord veins (Jaffe et al., 1973) and grown in M199 medium supplemented with 20% heat-inactivated human serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). All the studies were performed on second-passage human umbilical cord endothelial cells (HUVE). A primary keratinocyte cell line isolated from human foreskin was obtained from Clonetics Corporation (Boulder, Colo.) and maintained in a defined keratinocyte growth medium (Tsao et al., 1982). PBMC were isolated from venous blood fractionated on a Ficoll-Hypaque gradient. Monocytes were isolated from these PBMC by Percoll gradient fractionation (Wright and Silverman, 1982) and maintained in α-modified Eagle's medium (αMEM) supplemented with 10% heat-inactivated autologous human serum or fetal calf serum (FCS), penicillin, and streptomycin. FS4 cells were grown in αMEM supplemented with 10% FCS, penicillin, and streptomycin.

All induction experiments were performed in the regular cell growth media, using cells just before reaching confluence. Monocytes were induced at 10^6 cells/ml in Teflon dishes.

IFN-γ

The IFN-γ was a highly purified recombinant protein synthesized in *E. coli* generously provided by Genentech, Inc., San Francisco, Calif. The endotoxin levels were determined in a limulus amoebocyte lysate assay before shipping. The human rIFN-γ had a specific activity of $2–4\times10^7$ U/mg

as determined in a human lung carcinoma A549 inhibition assay using the encephalomyocarditis virus.

Peptide Synthesis

The peptide was synthesized by the solid-phase method (Barany and Merrifield, 1979) using chloromethylated, 1% crosslinked, styrene-divinylbenzene copolymer (Merrified Resin). Deprotection was followed by a coupling program that used the symmetric anhydrides of the appropriate Boc amino acids (Bachem Inc., Torrance, Calif.; or Peninsula Laboratories, Inc., Belmont, Calif.) (Yamashiro and Li, 1978). The side-chain functionalities were protected by benzyl-type protecting groups. The peptide was cleaved from the resin and deprotected by treatment with liquid HF-anisole at ~0° C. (Scotchler et al., 1970). The peptide was purified by gel-permeation chromatography, ion-exchange chromatography, and reverse-phase HPLC. The final product was homogeneous by analytical reverse-phase HPLC.

The peptide was glutaraldehyde coupled to keyhole limpet hemocyanin (KLH) (Pfaff et al., 1982). 2 μg of peptide was dissolved 10 μl of H_2O and added to 15 ng KLH in 2 ml 0.1M PBS. Glutaraldehyde (21 mM) was added over 1 h at room temperature. The mixture was allowed to stand overnight at room temperature and then was dialyzed against PBS.

Approximately 100 μg of protein in CFA was used to immunize two 8-wk-old female New Zealand white rabbits. The rabbits were boosted twice at 1-mo intervals with the same amount of protein in IFA. 10 d after the second boost, the rabbit was bled and serum was isolated and used for Western blotting and immunoprecipitation.

E. coli Expression and Production of Antiserum

A 576-bp Xba I-Eco RI insert derived from the IP-10 cDNA plasmid pIFNγ-31.7 (Matsuura, 1987) was cloned into the fusion expression vector $pB4^+$ (Leammli, 1970). The $pB4^+$ vector contains the gene for the influenza vital protein NSI. The plasmid was transformed into the *E. coli* strain AR58, which is a λ lysogen containing a temperature-sensitive mutation in the CI gene (CI857). The resulting strain, harboring the recombinant expression plasmid, produced a fusion protein at the nonpermissive temperature that consisted of 81 amino acids of NSI and 72 amino acids of IP-10.

A 325-bp Fnu4H fragment derived from the IP-10 cDNA plasmid pIFNγ 31.7 was cloned into the nonfusion expression vector pT17. This recombinant plasmid was transformed into the *E. coli* strain AR58, which upon temperature induction synthesized 88 amino acids of the IP-10 protein.

Bacterial cells were grown in L-broth at 30° C. to an OD_{650} of 0.3, then shifted to 42° C. for 1 h of growth. The bacterial cells were pelleted by centrifugation and resuspended in 1/20 vol of PBS. The cells were lysed by sonication and subjected to centrifugation at 10,000 rpm for 5 min. The pellet, which included the recombinant proteins and cellular envelopes, was suspended in sample buffer and subjected to preparative SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R for 1 min and immediately washed five times in deionized water. The faintly stained band was excised from the gel with a razor blade and minced. The gel pieces were soaked for 24 h in 50 mM Tris, pH 7.5, containing 0.15M NaCl and 0.1% SDS. The amount of eluted protein recovered was estimated by comparison to protein standards (Bio-Rad Laboratories, Richmond, Calif.) after SDS-PAGE. This treatment of the sample resulted in a preparation highly enriched for recombinant proteins.

Approximately 100 μg of protein in CFA was used to immunize an 8-wk-old female New Zealand white rabbit. The rabbit was boosted twice at 1-mo intervals with the same amount of protein in IFA. 10 d after the second boost, the rabbit was bled and serum was isolated and used for Western blotting and immunoprecipitation.

IgG was isolated from serum by protein A-Sepharose affinity chromatography (Pharmacia Fine Chemicals, Piscataway, N.J.). 1 mg of the gel-purified recombinant protein was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals). 100–200 mg IgG was affinity purified on the recombinant protein column. The bound IgG was eluted with 1M glycine-HCl, pH 2.8, quickly neutralized with 2M Tris, and dialyzed against PBS. The affinity-purified antiserum was used for immunoprecipitation, immunofluorescence, and immunohistochemistry.

Pulse-chase Experiments

HUVE cell monolayers (~10^6 cells) were washed twice with PBS and maintained for 30 min in αMEM lacking methionine but containing 200 mM glutamine. Cells were then pulsed for 30 min in this cell-starvation medium supplemented with [^{35}S]methionine (500 μCi/ml). After removal of the pulse medium, the cells were washed twice with PBS and then incubated with cell growth medium supplemented with unlabeled methionine (5 mM) for the chase periods indicated. At the completion of the chase, the monolayers were washed twice with PBS and scraped into an SDS solution (0.5% SDS, 50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM EDTA). After being heated for 2 min at 100° C., the samples were frozen at −20° C. For immunoprecipitation, the samples were again heated for 2 min at 100° C., sonicated for 2 min, and adjusted to contain 0.2 U/ml aprotinin (Sigma Chemical Co., St. Louis, Mo.), and 1 mM PMSF (Sigma Chemical Co.). Affinity-purified antibodies were added to a final concentration of 5 μg/ml and the immunoprecipitation was continued as described below.

Immunoblotting

E. coli cells or human cell lysates were dissolved in sample buffer (2% SDS/0.0625M Tris, pH 7.4/10% glycerol/0.01% bromphenol blue/and 5% 2-ME), boiled for 5 min, and fractionated by SDS-PAGE using slab gels of 12.5 and 15% acrylamide. Prestained protein molecular weight standards (Bethesda Research Laboratories, Gaithersburg, Md.) were used to calculate apparent molecular weights. Protein was transferred electrophoretically to nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.). All of the following steps were performed in Tris-buffered saline (TBS; 50 mM Tris, pH 7.5, 2 mM EDTA, 0.15M NaCl), 0.5% NP-40, and 0% FCS. The nitrocellulose filter was first treated overnight in 5% nonfat dry milk, followed by incubation for 2 h with a 1:1,000 dilution of antiserum. The nitrocellulose was washed and reacted for 1 h with 10^6 cpm/ml of ^{125}I Staphylococcus protein A (Amersham Corp., Amersham, Arlington Heights, Ill.). After extensive washing in TBS+0.5% NP-40, the filter was exposed to x-ray film at −70° C. in the presence of an intensifying screen.

Immunoprecipitation

Approximately 10^6 cells were lysed in PBS containing 1% NP-40, 0.2 U/ml aprotinin, 1 mM PMSF, and 0.1% diisopropylfluorophosphate (Sigma Chemical Co.). Nuclei and debris were removed by centrifugation at 14,000 g for 5 min. The lysate was adjusted to 0.2% SDS and boiled for 5 min at 100° C. The lysate was further clarified by centrifugation at 45,000 g, 15 min at 4° C. The supernatant was passed through a 0.45-μm millex filter and further clarified by centrifugation at 45,000 g, 15 min at 4° C. Affinity-purified antibodies were added to a final concentration of 5 μg/ml to both lysate and supernatant. The

solution was incubated at room temperature for 4–16 h. Antigen-antibody complexes were precipitated by incubation with protein A-Sepharose for 2 h at room temperature.

The immunoabsorbed protein A-Sepharose beads were collected by centrifugation, washed twice in buffer that contained 0.6M NaCl, 0.0125M KPO_4, pH 7.4, and 0.02% NaN_3 (HSA buffer), twice at room temperature with a mixed detergent solution (0.05% NP40, 0.1% SDS, 0.3M NaCl, and 10 mM Tris-HCl, pH 8.6), once again with HSA buffer, and finally, once in PBS. The antigen-antibody complexes were released from the beads by incubation at 100° C. for 2 min in 2× PAGE sample buffer.

NH_2-Terminal Sequence Determination

Human keratinocytes were biosynthetically labeled with [^{3}H]leucine and [^{35}S]cystein for 8 h. Radiolabeled IP-10 was purified from the keratinocyte media by immunoprecipitation and SDS-PAGE. The gels were dried down without fixing or staining. The IP-10 protein was located in the gel by autoradiography, electroeluted from the gel, and concentrated by precipitation (Schragger, 1987). Samples were subjected to automated Edman degradation in a Gas Phase Sequencer (Model 470A; Applied Biosystems, Inc., Foster City, Calif.). The amino acid derivative obtained at each cycle was dissolved in 20% acetonitrile and transferred to 10 ml of Aquasol for scintillation counting.

ELISA

The antipeptide antisera were checked for their ability to react with the synthetic peptide by an ELISA assay. Microtiter plates were coated with peptide (100 ng/well) in a sodium carbonate buffer, pH 9.6, and then saturated with 3% BSA, PBS, 0.1% Tween 20. One hundred microliters of antipeptide antisera dilutions from 10^{-2} to 10^{-7} were placed in the wells and incubated for 2 h. After extensive washing in PBS/0.05% Tween, the plates were incubated for 2 h with peroxidase-labeled affinity-purified goat anti-rabbit IgG. The wells were then washed and the substrate O-phenylenediamine (100 µl of 10 mg/25 ml in 0.05M citrate phosphate buffer, pH 5) was added. The reaction was allowed to proceed for 10 min at room temperature and was stopped by addition of 50 ml of 2.5M H_2SO_4. Absorbance was read at 410 nm in an automatic plate reader (Minireader II; Dynatech Laboratories, Inc., Alexandria, Va.). Positive ELISA readings were obtained at dilutions of 10^{-7} after boosting with peptide conjugate. The purified peptide was attached to CNBr-activated Sepharose 4B. This matrix was used to affinity purify the rabbit antipeptide antiserum. The affinity-purified antibodies were used for Western blotting and immunoprecipitation experiments.

RNA Isolation and Blotting

Total cellular RNA was isolated by the guanidinium isothiocyanate-cesium chloride method. RNA was fractionated on a 1% agarose gel containing 2.2M formaldehyde (Irie, 1980) and transferred to nitrocellulose (Michaels, 1991) and hybridized with a random primed (Towbin, 1979) IP-10 cDNA probe (pIFNγ-31.7). Hybridization was performed at 40° C. for 16 h in a solution containing 50% formamide, 10% dextran sulfate, 5× SSC (1× SSC: 0.15M sodium chloride, 0.015M sodium citrate), 1× Denhardt's (0.02% polyvinyl-pyrrolidone, 0.02% Ficoll, and 0.02% BSA), and 200 µg/ml of sonicated herring sperm DNA. The filters were washed at 50° C. in 0.1× SSC containing 0.1% SDS and exposed at −70° C. to Kodak XAR film in the presence of one intensifying screen (Cronex Lightning Plus).

EXPRESSION OF RECOMBINANT IP-10

Recombinant IP-10 has been expressed in both insect and bacterial systems as described in Examples II and III. The IP-10 secreted from insect cells appears to be lacking the signal sequence, which is also removed from the naturally occuring protein which is secreted from keratinocytes, for example. In addition, the full length protein and two smaller fragments have been produced by a bacterial expression system.

EXAMPLE II

Expression in Insect Cells

Materials and Methods

All chemicals were reagent grade. Protein molecular weight markers were from BRL (Gaithersburg, Md.). Ficoll-Hypaque, Protein A-Sepharose, and S-Sepharose were from Pharmacia (Piscataway, N.J.). EX-CELL-400 was from JR Scientific (Woodland, Calif.). The 4.6×150 mm C4 reverse phase column was from Vydac (Hesperia, Calif.). The Rapid-Ag Silver protein staining kit was from ICN (Cleveland, Ohio).

Wild type baculovirus, Sf9 insect cells, and transfer vector pVL1392 (Webb and Summers, 1990) were provided by M D Summers (Texas A&M University, College Station, Tex.). Transfer vector pAcYM1 (Matsuura et. al., 1987) was provided by D. Bishop (NERC Institute of Virology, Oxford, UK). The Pst-1 fragment of the IP-10 cDNA was cloned in the Pst-1 site of pVL1392, and yielded recombinant baculoviruses 8555 and 9094. For elimination of its 5' untranslated sequences, the IP-10 cDNA was digested with Nla3, the 375 nucleotide fragment was purified, ligated to GATCCATG, restricted with BamH1, and cloned into the BamH1 site of pAcYM1, generating recombinant baculoviruses A213 and A221. Standard techniques were performed for isolation of recombinant baculoviruses (Webb and Summers, 1990; Sambrook et. al., 1989). All junctions between recombinant transfer vectors and IP-10 cDNA were sequenced (Sanger et. al., 1987) to exclude cloning artifacts. For production of rIP-10, Sf9 cells were infected with A221 (20 PFU/cell), 6-day supernatants were cleared at 100,000 G for 1 h at 4° C., dialyzed against 40 mM Na Phosphate pH 7.2, and loaded on a 15 ml Sepharose-S FPLC column. Proteins were eluted with a linear gradient of 0.0–2.0M NaCl in 40 mM Na Phosphate pH 7.2 (150 ml), fractions containing rIP-10 were identified with a dot blot immunoassay (Sarris et. al., 1992) using anti-IP-10, and were loaded on a reverse-phase C4 HPLC column. Adsorbed rIP-10 was eluted with a gradient of 25% acetonitrile-50% propanol-25% H_2O in 0.1% TFA, lyophilized and resuspended in endotoxin-free PBS.

Proteins were separated on 10–20% gradient PAGE in the presence of 0.1% SDS with 0.75M Tris-HCl, pH 8.45, in the stacking gel; 1.0M Tris-HCl, pH 8.45, in the separating gel; 0.2M Tris HCl, pH 8.9 in the anode reservoir; and 0.1M Tris-0.1M Tricine in the cathode reservoir (Schrågger and von Jagow, 1987). Protein gels were stained with silver (Irie, 1980), and quantitated by densitometry. Western blots were performed on Immobilon-P (Sarris et. al., 1992; Towbin et. al., 1979). rIP-10 was immunoprecipitated from Sf9 cells labeled with [^{35}S]-methionine after boiling in 500 µl of 0.2% SDS, 50mM Tris-HCl, pH 7.5, and the sequential addition of NP-40 (final concentration of 1%), affinity purified antibodies (final concentration 5 µg/ml), and Protein-A beads (Sarris and Palade, 1982). The M_r of IP-10 was estimated from the mobilities of marker proteins of 43–14.3 kDa. Amino-terminal sequencing was performed as described (Hewick et. al., 1981; Tempst and Riviere, 1989).

Results

Recombinant baculoviruses 8555, 9094, A213 and A221 express rIP-10 as a nonfusion protein. In baculovirus 8555 the initiating codon, ATG of the polyhedrin gene is mutated to ATT, and protein synthesis starts 118 nucleotides downstream, at the initiating ATG of the IP-10 cDNA. In baculovirus 9094 the IP-10 was inserted in the same location but in reverse orientation. In A221 and A213 the initiating ATG of the polyhedrin gene is destroyed by deletion of nucleotides 2–751 of its coding region, and protein synthesis starts 8 nucleotides downstream at the initiating ATG of IP-10. Most 3'-untranslated sequences of the IP-10 were deleted from A213 and A221.

Analysis of protein synthesis after infection by 8555 and A221 demonstrated a major new band of 9.9 kDa, similar in size to IP-10 without the signal sequence (10.0 kDa), and a minor band of 11.9 kDa, similar in size to IP-10 with the signal sequence (12.4 kDa). Neither band was detected in uninfected cells or in cells infected with 9094 or with wild type virus (FIG. 1A). The 9.9 kDa but not the 11.9 kDa band was detected in supernatants of infected cells by autoradiography. Non-immune serum did not precipitate any proteins from cells infected with A221, but anti-IP-10 and anti-22 precipitated both bands (FIG. 1B). Western blotting with anti-IP-10 and anti-22 detected both bands in cells, but only the 10 kDa band in the media.

In order to precisely define the M_r of baculovirus IP-10, f(22–98) and f(22–77) were used as molecular weight markers. These studies demonstrated that f(22–98) and IP-10 derived from either keratinocytes or baculovirus co-migrated at 10.2 kDa, and were recognized by anti-IP-10 and anti-22. Alternatively, f(22–77) migrated with an apparent M_r of 6.2 kDa, and was recognized by anti-IP-10 but not by anti-22 (FIG. 2). The reactions of antiserum AS522 and anti-IP-10 in immunoprecipitations and western blots were identical.

Levels of rIP-10 were 5–10 times higher after infection with A213 and A221 than after infection with 8555, and were not affected by FCS reaching 9% of the total protein in the supernatant of cells grown in EXCELL-400. Anti-IP-10 and a dot blot immunoassay were used to purify rIP-10 from supernatants of infected cells, and a major HPLC peak was obtained (FIG. 3A) which was a single band on SDS-PAGE (FIG. 3B, lanes S, F, H) co-migrating with purified bacterial f(22–98) (FIG. 3B, lane E). The faint bands near the top of the gels correspond in size to keratins, and were seen in unloaded lanes and in lanes loaded only with sample buffer. Western blotting confirmed that the purified band represented rIP-10, because it reacted with anti-IP-10 and anti-22 during all stages of purification (FIG. 2).

Aminoterminal sequencing of baculovirus rIP-10 demonstrated a major amino terminal sequence of VPLSRTVRØT SEQ ID NO:4 (66%) and a minor sequence of RTVRØT SEQ ID NO:5 (34%), both matching the sequence of IP-10 secreted by keratinocytes (Luster et al., 1987). The purified baculovirus IP-10 thus appears to be identical to natural IP-10. The amino acid sequence of the major species is V P L S R T V R C T C I S I S N Q P V N P R S L E K-LEIIPASQFCPRVEIIATMKKKGEKRCLNPESKAIKNLL KAVSKEMSKRSP (residues 2–78 SEQ ID NO:6, i.e. lacking the N-terminal Methionine). The yield of purified rIP-10 was 0.5 μg/ml of supernatant in the baculovirus system.

EXAMPLE III

Expression in Bacterial Cells

This section describes the generation and purification of two forms of recombinant IP-10; f(22–98) which is identical to the naturally occurring IP-10 but has an added methionine at its amino terminal, and f(22–77) which differs from f(22–98) by a deletion of the last 21-carboxy-terminal amino acids.

Using two primers, A (GGATCCATGGTACCTCTCTCTAGAACC; seq id no:1) as 5' and B (GGATCCATGGTTAAGGAGATCTTTTAGA; seq id no:2) as 3' primer, a cDNA coding for f(22–98) was amplified (Saiki et. al., 1988). This segment, f(22–98) is a fragment lacking the signal peptide and extending from valine 22 to proline 98 of IP- 10. With C (GGATCCATGGTTATGGATTCAGACATCTCTT; seq id no:3) as 3' and A as the 5' primer a cDNA coding for f(22–77) was also amplified. This segment encodes a fragment extending from valine 22 to proline 77 and lacking the signal peptide and the last 21 residues of IP-10, but retaining all 4 cysteine residues and approximating the previously reported, secreted form of IP-10 (Luster et al., 1987). The PCR products were restriction digested with NcoI and cloned in the NcoI site of pET-3d (provided by F. Studier, Brookhaven National Laboratories, New York) eliminating all amino acid residues of ϕ10 (Studier et. al., 1990), and expressing the rIP-10 fragments as nonfusion proteins with an added methionine at the amino terminus. The regions adjacent to and including the IP-10 cDNA were sequenced (Sanger et al., 1987) to exclude PCR and cloning errors. Lysogens BL21(DE3) transformed with recombinant plasmids were induced with 0.4 mM isopropylthiogalactoside (Studier et al., 1990), recombinant f(22–98) was purified (Lindley et al., 1988) from refractile bodies (Marston), dialysed against Ca/Mg-free PBS, and stored in 1 ml aliquots at –70° C. Protein concentration was measured by dye binding (Bradford, 1976).

Sequencing of f(22–98) demonstrated a single amino-terminal sequence of MVPLSRTVROTOISISNQPVN SEQ ID NO:7 matching the sequence of secreted IP-10 (Luster et al., 1987) with an additional amino-terminal methionine. The yield of bacterial IP-10 [f(22–98)] was 5 μg/ml of bacterial culture. The sequences of bacterial IP-10 were: f(22–98)

MVPLSRTVRCTCISISNQPVNPRSLEKLEIIPASQFCPRVEIIATMKKKGEKRCLNPESKAIKNLLKAVSKEMSKRSP	(SEQ ID NO: 8); and f(22–77) (residues 1–57 of SEQ ID NO: 9).
MVPLSRTVRCTCISISNQPVNPRSLEKLEIIPASQFCPRVEIIATMKKKGEKRCLNP	

EXAMPLE IV

Colony Suppression by IP-10

Material and Methods

rIFN-γ was a gift from Dr G. Garotta (Hoffman-LaRoche, Basel Switzerland). Human rMIP-1α and rMIP-2α were gifts of Dr B. Sherry (Picower Institute, Manhasset, N.Y.). Human rGM-CSF, human rIL-3 and human rSLF were gifts of the Immunex Corporation (Seattle, Wash.). Human rEPO was purchased from Amgen (Thousand Oaks, Calif.), human

platelet Factor 4 (PF4) and rIL-8 from Sigma (St Louis, Mo.), and human rMCAF from Repro Tech Inc, Rocky Hills, N.J.

Human bone marrow CFU-GM, BFU-E, and CFU-GEMM of healthy volunteers were assayed as described (Broxmeyer et al., 1990; Broxmeyer et. al., 1991; Broxmeyer et. al., 1993). rSLF (50 ng/ml) and other recombinant or natural chemokines were added as indicated. Highly purified CD-34^{+++} cells (Broxmeyer et al., 1990; Broxmeyer et. al., 1993) were plated (500 cell/ml) in the presence of rEPO (1 U/ml), rSLF (50 ng/ml), rGM-CSF (100 U/ml) and rIL-3 (200 U/ml), and yielded 126±10, 60±10 and 17±5 CFU-GM, BFU-E and CFU-GEMM per 500 plated cells, respectively. For reversal of inhibition, chemokines were pre-incubated with a 4-fold molar excess of affinity purified antibodies (anti-IP-10 or anti-22) or with whole serum (AS522) in McCoy's media at room temperature for 1 h. The mixture was added to human bone marrow cells and CFU-GM were assayed in McCoy's media supplemented with FCS, rSLF (50 ng/ml) and GM-CSF (100 U/ml). For controls, an equal volume of similarly incubated McCoy's medium was added to cells. Statistical significance was determined with the Student's two-tailed test.

Results

The effect of rIP-10 on colony formation by CFU-GM, BFU-E and CFU-GEMM was evaluated. rIP-10 (50 and 500 ng/ml) did not affect colony formation by marrow cells plated in media alone or in the presence of single growth factors (rEPO or rGM-CSF). However, rIP-10 suppressed colony formation of CFU-GM stimulated by rGM-CSF and rSLF, and BFU-E and CFU-GEMM stimulated by rEPO and rSLF. Concentrations of 1–10 ng/ml were inactive, but there was a dose dependent inhibition between 25 and 50 ng/ml. Maximal inhibition (50–60%) was seen at 50–500 ng/ml of rIP-10 (FIG. 4), representing complete suppression of the additional CFU-GM and BFU-E or CFU-GEMM colonies generated by-the respective addition of rSLF to rGM-CSF or rEPO. The dose response was similar to that of rMIP-1α, rMIP-2α, PF4, rIL-8, or rMCAF (Broxmeyer et. al., 1991; Broxmeyer et. al., 1993) which were assessed in the same assays. Whereas individual chemokines were inactive at concentrations <10 ng/ml, significant suppression of colony formation ($p<0.01$) was seen when 0.01 ng/ml of rIP-10 was combined with 0.01 ng/ml of rMIP-1α, rMIP-2α, PF4, rIL-8, or rMCAF. Combinations of 0.1 ng/ml of rIP-10 with 0.1 ng/ml of any of these chemokines resulted in a 50–60% inhibition of colony formation by CFU-GM, BFU-E and CFU-GEMM ($p<0.001$). This represented complete inhibition of the rSLF-dependent colonies, and could not be suppressed further with combination of 50 ng/ml of rIP-10, rMIP-1α, rMIP-2, PF4, rIL-8, and rMCAF (FIG. 4). In the presence of rIP-10 (100 ng/ml) colony formation by CD-34^{+} $^{++}$ cells was inhibited by 77% for CFU-GM ($p<0.01$), by 58% for BFU-E ($p<0.05$), and by 82% for CFU-GEMM ($p<0.05$).

The inhibitory activity of rIP-10 but not that of rMIP-1α or PF4 was neutralized by antibodies raised against whole IP-10 (anti-IP-10 and AS522). Antisera raised against the 22 carboxy-terminal residues of IP-10 (anti-22) did not affect the inhibitory activity of rIP-10, rMIP-1α, or PF4. These antibodies had no effect on colonies grown in the absence of rIP-10 (Table 1).

TABLE 1

Antibody against IP-10 neutralizes its ability to suppress CFU-GM in vitro.

	Colony Formation after Preincubation with:			
	media	anti-IP-10	AS522	anti-22
Chemokine media	61 ± 1	64 ± 1 (+2)	61 ± 1 (0)	63 ± 2 (+3)
IP-10	34 ± 3(−44)*	62 ± 1(+2)	61 ± 2(0)	32 ± 2 (−48)*
MIP-1α	33 ± 2 (−46)*	32 ± 3 (−48)*	37 ± 2 (−39)*	33 ± 2 (−46)*
PF4	31 ± 1 (−49)*	34 ± 2 (−44)*	36 ± 1 (−41)*	33 ± 2 (−48)*

CFU-GM grown with GM-CSF (100 U/ml) and SLF (50 ng/ml) are expressed as mean ± 1 SEM per 10^3 plated cells.
Chemokines were used at 50 ng/ml.
Purified f(22-98) was the source of rIP-10.
Values in parentheses designate percent inhibition relative to control.
*designates significant decrease ($p < 0.001$) relative to control.

While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

Barany, G. and R. B. Merrifield. 1979. Solid phase peptide synthesis. In The Peptides. Vol. II. E. Gross and J. Meinhoper, editors. Academic Press, New York. 1–284.

Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72:248.

Broxmeyer, H. E, B Sherry, S. Cooper, L. Lu, R. Maze, M. P. Beckmann, A. Cerami and P. Ralph. 1993. Comparative analysis of the human macrophage inflammatory protein family of cytokines (chemokines) on proliferation of human myeloid progenitor cells: interacting effects involving suppression, synergistic suppression and blocking of suppression. *J Immunol*, 150:3448.

Broxmeyer, H. E., B. Sherry, S. Cooper, F. W. Ruscetti, D. E. Williams, P. Arosio, B. S. Kwon, and A. Cerami. 1991. Macrophage inflammatory protein (MIP)-1b abrogates the capacity of MIP-1α to suppress myeloid progenitor cell growth. *J Immunol.*, 147:2586.

Broxmeyer, H. E., B. Sherry, L. Lu, S. Cooper, K.-O. Oh, P. Tekamp-Olson, B. S. Kwon, and A. Cerami. 1990. Enhancing and suppressing effects of recombinant murine macrophage inflammatory proteins on colony formation in vitro by bone marrow myeloid progenitor cells. *Blood*, 76:1110.

Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry*. 18:5294.

Dimitriadis, G. J. 1979. Effect of detergents on antibody-antigen interaction. *Anal Biochem,* 98:445.

Dunlop, D. J., E. G. Wright, S Lorimore, G. J. Graham, T. Holyoake, D. J. Kerr, S. D. Wolpe, and I. B. Pragnell. 1992. Demonstration of stem cell inhibition and myelo-protective effects SCI/rhMIP1a in vivo. *Blood,* 79:2221.

Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6.

Graham, G. J., E. G. Wright, R. Rewick, S. D. Wolpe, N. M. Wilkie, D. Donaldson, S. Larimore, and I. B. Pragnell. 1990. Identification and characterization of an inhibitor of hematopoietic stem cell proliferation. *Nature,* 344:442.

Hewick, R. M., M. W. Hunkapiller, L. E. Hood, and W. J. Dreyer. 1981. A gas-liquid solid phase peptide and protein sequenator. *J Biol Chem,* 256:7990.

Irie, S. 1980. A highly sensitive silver staining for detection of proteins in polyacrylamide gels. *Biochemistry* (Japan), 52:411.

Jaffe, E. A., R. C. Nachman, C. G. Becker, and C. R. Minick. 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest.* 52:2745.

Kahn, J., Ruiz, R., Kerschmann, R., Berger, T., Ma, R., Coleman, R., Alford, B. and L. Kaplan. 1993. A phase ½ study of recombinant platelet factor 4 (rPF4) in patients with AIDS related Karposi's Sarcoma, *Proc Amer Soc Clin Oncology* 12:4a.

Lehrach, H., D. Diamond, J. M. Worney, and H. Boedtler. 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry.* 16:7443.

Lindley, I., H. Aschauer, J. M. Seifert, C. Lam, W. Brunowsky, E. Kownatzki, M. Thelen, P. Peveri, B. Dewald, V. von Tscharner, A. Waltz, and M. Baggiolini. 1988. Synthesis and expression in *Escherichia coli* of the gene encoding monocyte-derived neutrophil-activating factor: Biological equivalence between natural and recombinant neutrophil-activating factor. *Proc Natl Acad Sci USA,* 85:9199.

Lord, B. I., T. M. Dexter, J. M. Clements, M. A. Hunter and A. J. H. Gearing. 1992. Macrophage inflammatory protein protects multipotent hematopoietic cells from the cytotoxic effects of hydroxyurea in vivo. *Blood,* 79:2605.

Luster, A. D., and J. V. Ravetch. 1987. Biochemical characterization of a g Interferon-inducible cytokine (IP-10). *J Exp Med,* 166:1084.

Luster, A. D., J. C. Unkeless, and J. V. Ravetch. 1985. g-Interferon transcriptionally regulates an early-response gene containing homology to platelet proteins. *Nature,* 315:672.

Marston, F. A. O. The purification of eukaryotic polypeptides expressed in *Escherichia coli.* In "DNA Cloning: A Practical Approach" (Ed M Glover) vol 3, pp59, IRL Press, Oxford.

Matsuura, Y., R. D. Possee, H. A. Overton, and D. H. Bishop. 1987. Baculovirus expression vectors: the requirements for high level expression of proteins, including glycoproteins. *J Gen Virol,* 68:1233.

Maze, R., B. Sherry, B. S. Kwon, A. Cerami, and H. E. Broxmeyer. 1992. Myelosuppressive effects in vivo of purified recombinant murine macrophage inflammatory protein-1a. *J. Immunol,* 149:1004.

Oppenheim, J. J., C. O. C. Zachariae, N. Mukaida, and K. Matsushima. 1991. Properties of the novel proinflammatory supergene "intercrine" cytokine family. *Ann Rev Immunol.* 9:61.

Pfaff, E. P., M. Mossgay, H. B. Bohm, G. E. Schulz, and H. Schuller. 1982. Antibodies against a preselected peptide recognize and neutralize foot and mouth disease virus. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:869.

Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Elrich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science,* 239:487.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual. Second edition.* Cold Spring Harbor Laboratory Press.

Sanger, F., S. Nickelson, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA,* 74:5463.

Sarris, A. H., M. W. Harding, T. R. Jiang, D. Aftab, and R. E. Handschumacher. 1992. Immunofluorescent localization and immunochemical determination of Cyclophilin-A with specific rabbit antisera. *Transplantation,* 54:904.

Sarris, A. H., and G. E Palade. 1982. Immunofluorescent detection of erythrocyte sialoglycoprotein antigens on murine erythroid cells. *J Cell Biol.* 93:591.

Schrågger, H., and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem,* 166:368.

Scotchler, J., R. Lozier, and A. B. Robinson. 1970. Cleavage of single amino acid residues from purified resin with hydrogen chloride and hydrogen fluoride. *J. Org. Chem.* 35:3151.

Shatzman, A., Y-S. Ho, and M. Rosenberg. 1983. Use of phage lambda regulatory signals to obtain efficient expression of genes in *E. coli.* In Experimental Manipulation of Gene Expression. Academic Press, New York. 1–14.

Shields, D., and G. Blobel. 1977. Cell free synthesis of fish preproinsulin, and processing by heterologous mammalian microsomal membranes. *Proc. Natl. Acad. Sci. USA.* 74:2059.

Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503

Studier F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff. 1990. Use of T7 polymerase to direct expression of cloned genes. *Methods in Enzymology,* 185:60.

Tempst, P., and L. Riviere. 1989. Examination of automated polypeptide sequencing using standard phenyl isothiocyanate reagent and subpicomole high-performance liquid chromatographic analysis. *Anal Biochem,* 183:290.

Tsao, M. C., B. J. Walthall, and R. C. Ham. 1982. Clonal growth of normal human epidermal keratinocytes in a defined medium. *J. Cell. Physiol.* 110:219.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitro-cellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA,* 76:4350.

Webb, N. R. and M. D. Summers. 1990. Expression of proteins using recombinant baculoviruses. *Technique,* 2: 173.

Wolpe, S. D., and A. Cerami. 1989. Macrophage inflammatory proteins 1 and 2: members of a novel superfamily of cytokines. *FASEB J,* 3:2565.

Wright, S. D., and S. C. Silverstein. 1982. Tumor-promoting phorbol esters stimulate C3b and C3b' receptor-mediated phagocytosis in cultured human monocytes. *J. Exp. Med.* 156:1149.

Yamashiro, D., and C. H. Li. 1978. Total synthesis of ovine β lipotropin by the solid-phase method. *J. Am. Chem. Soc.* 100:5174.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 9

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i x) SEQUENCE DESCRIPTION: SEQ ID NO:1:
GGATCCATGG TACCTCTCTC TAGAACC

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i x) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGATCCATGG TTAAGGAGAT CTTTTAGA 28

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i x) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGATCCATGG TTATGGATTC AGACATCTCT T 31

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i x) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Val Pro Leu Ser Arg Thr Val Arg Xaa Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i x) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Arg Thr Val Arg Xaa Thr
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 77 amino acid residues
(B) TYPE: amino acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i x) SEQUENCE DESCRIPTION: SEQ ID NO:6:

-continued

```
Val Pro Leu Ser Arg Thr Val Arg Cys Thr Cys Ile Ser Ile Ser Asn
 1               5                  10                  15

Gln Pro Val Asn Pro Arg Ser Leu Glu Lys Leu Glu Ile Ile Pro Ala
            20                  25                  30

Ser Gln Phe Cys Pro Arg Val Glu Ile Ile Ala Thr Met Lys Lys Lys
        35                  40                  45

Gly Glu Lys Arg Cys Leu Asn Pro Glu Ser Lys Ala Ile Lys Asn Leu
    50                  55                  60

Leu Lys Ala Val Ser Lys Glu Met Ser Lys Arg Ser Pro
65                  70                  75
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i x) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Met Val Pro Leu Ser Arg Thr Val Arg Xaa Thr Xaa Ile Ser Ile
 1               5                  10                  15

Ser Asn Gln Pro Val Asn
            20
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 78 amino acid residues
(B) TYPE: amino acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i x) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Met Val Pro Leu Ser Arg Thr Val Arg Cys Thr Cys Ile Ser Ile Ser
 1               5                  10                  15

Asn Gln Pro Val Asn Pro Arg Ser Leu Glu Lys Leu Glu Ile Ile Pro
            20                  25                  30

Ala Ser Gln Phe Cys Pro Arg Val Glu Ile Ile Ala Thr Met Lys Lys
        35                  40                  45

Lys Gly Glu Lys Arg Cys Leu Asn Pro Glu Ser Lys Ala Ile Lys Asn
    50                  55                  60

Leu Leu Lys Ala Val Ser Lys Glu Met Ser Lys Arg Ser Pro
65                  70                  75
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 amino acid residues
(B) TYPE: amino acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i x) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Met Val Pro Leu Ser Arg Thr Val Arg Cys Thr Cys Ile Ser Ile Ser
 1               5                  10                  15

Asn Gln Pro Val Asn Pro Arg Ser Leu Glu Lys Leu Glu Ile Ile Pro
            20                  25                  30
```

-continued

Ala Ser Gln Phe Cys Pro Arg Val Glu Ile Ile Ala Thr Met Lys Lys
35 40 45

Lys Gly Glu Lys Arg Cys Leu Asn Pro
50 55

What is claimed is:

1. A method for inhibiting the growth of hematopoietic progenitor cells comprising contacting a population of cells that include hematopoietic progenitor cells with an effective amount of IP-10.

2. The method of claim 1, wherein the IP-10 comprises recombinant IP-10.

3. The method of claim 2, wherein the recombinant IP-10 comprises f(22–98) IP-10.

4. The method of claim 1, wherein IP-10 is prepared by a process that includes:

(a) preparing a recombinant host cell comprising a recombinant gene segment encoding IP-10;

(b) culturing said cell under conditions effective to allow expression of said gene to produce IP-10; and

(c) collecting and purifying the IP-10 so produced.

5. The method of claim 1, wherein the IP-10 is comprised in a pharmaceutical composition.

6. The method of claim 5, wherein the pharmaceutical composition is administered to a human cancer patient.

7. The method of claim 6, wherein a therapeutically effective amount of an antineoplastic agent having bone marrow toxicity is administered to the patient, in conjunction with administration of the IP-10 pharmaceutical composition.

8. An improved method for chemotherapy of a cancer patient employing an antineoplastic agent having bone marrow toxicity, wherein the improvement comprises treating said patient with a hematopoietic progenitor cell inhibitory amount of IP-10.

9. A method for inhibiting the growth of hematopoietic progenitor cells in a patient comprising the steps of:

(a) preparing a recombinant host cell comprising a recombinant gene segment encoding IP-10;

(b) culturing said cell under conditions effective to allow expression of said gene to produce IP-10;

(c) collecting and purifying the recombinant IP-10 so produced;

(d) rendering said recombinant IP-10 pharmacologically acceptable; and

(e) administering said pharmacologically acceptable IP-10 to said patient in an amount effective to inhibit hematopoietic progenitor cells.

10. The method of claim 9, wherein the IP-10 is produced from a baculovirus expression vector.

11. The method of claim 9, wherein the patient is a human cancer patient.

12. The method of claim 9, wherein the patient is administered an agent having bone marrow toxicity.

13. The method of claim 12, wherein the patient is a cancer patient, and the cancer patient is administered an antineoplastic agent having bone marrow toxicity.

14. The method of claim 9, wherein the recombinant gene segment encodes f(22–98) IP-10.

15. The method of claim 9, wherein the recombinant gene segment encodes full length IP-10.

* * * * *

US006110747A

United States Patent [19]

Blaschuk et al.

[11] **Patent Number: 6,110,747**

[45] **Date of Patent: Aug. 29, 2000**

[54] **COMPOUNDS AND METHODS FOR MODULATING TISSUE PERMEABILITY**

[75] Inventors: **Orest W. Blaschuk**, Westmount; **James Matthew Symonds**, Ottawa; **Barbara J. Gour**, Kemptville, all of Canada

[73] Assignee: **Adherex Technologies Inc.**, Ottawa, Canada

[21] Appl. No.: **09/222,373**

[22] Filed: **Dec. 29, 1998**

Related U.S. Application Data

[63] Continuation-in-part of application No. 09/001,511, Dec. 31, 1997.

[51] **Int. Cl.**[7] **G01N 33/563**

[52] **U.S. Cl.** **436/512**; 436/63; 436/86; 530/300; 530/317; 530/324; 530/325; 530/326; 530/327; 530/328; 530/329; 530/330

[58] **Field of Search** 436/512, 63, 86; 530/387, 300, 317, 324, 325, 326, 327, 328, 329, 330

[56] **References Cited**

FOREIGN PATENT DOCUMENTS

0831148	3/1998	European Pat. Off. .
831 148 A1	3/1998	European Pat. Off. .
WO 97/32982	9/1997	Japan .
9733605	9/1997	WIPO .
WO 97/33605	9/1997	WIPO .
9821237	5/1998	WIPO .
WO 98/21237	5/1998	WIPO .

OTHER PUBLICATIONS

Jaeger et al., "Small Synthetic Peptides Homologous To Segments Of Occludin Impair Tight Junction Resealing In A Ca^{+2} Switch Assay In A A6 Cell Monolayers," *Mol. Biol. Cell.* (Suppl.): p. 205A, Abstract No. 1189, 1997.

Pique et al., "Among All Human T–Cell Leukemia Virus Type 1 Proteins, Tax, Polymerase, and Envelope Proteins Are Predicted as Preferential Targets for the HLA–A2–Restricted Cytotoxic T–Cell Response," *Journal Of Virology* 70(8): 4919–4926, 1996.

Wong and Gumbiner, "A Synthetic Peptide Corresponding to the Extracellular Domain of Occludin Perturbs the Tight Junction Permeability Barrier," *Journal of Cell Biology* 136(2): 399–409, 1997.

Ando–Akatsuka et al., "Interspecies Diversity of the Occludin Sequence: cDNA Cloning of Human, Mouse, Dog, and Rat–Kangaroo Homologues," *The Journal of Cell Biology* 133(1): 43–47, 1996.

Chen et al., "COOH Terminus of Occludin Is Required for Tight Junction Barrier Function in Early *Xenopus* Embryos," *The Journal of Cell Biology* 138(4): 891–899, 1997.

Furuse et al., "Overexpression of occludin, a tight junction–associated integral membrane protein, induces the formation of intracellular multilamellar bodies bearing tight junction–like structures," *Journal of Cell Science* 109: 429–435, 1996.

Furuse et al., "Occludin: A Novel Integral Membrane Protein Localizing at Tight Junctions," *The Journal of Cell Biology* 123(No. 6, Part 2): 1777–1788, 1993.

Lampugnani and Dejana, "Interendothelial junctions: structure, signalling and functional roles," *Current Opinion in Cell Biology* 9: 674–682, 1997.

Primary Examiner—Avis M. Davenport
Attorney, Agent, or Firm—Seed IP Law Group PLLC

[57] **ABSTRACT**

Methods for using modulating agents to enhance or inhibit occludin-mediated cell adhesion in a variety of in vivo and in vitro contexts are provided. Within certain embodiments, the modulating agents may be used to increase vasopermeability. The modulating agents comprise at least one occludin cell adhesion recognition sequence or an antibody or fragment thereof that specifically binds the occludin cell adhesion recognition sequence. Modulating agents may additionally comprise one or more cell adhesion recognition sequences recognized by other adhesion molecules. Such modulating agents may, but need not, be linked to a targeting agent, drug and/or support material.

9 Claims, 12 Drawing Sheets

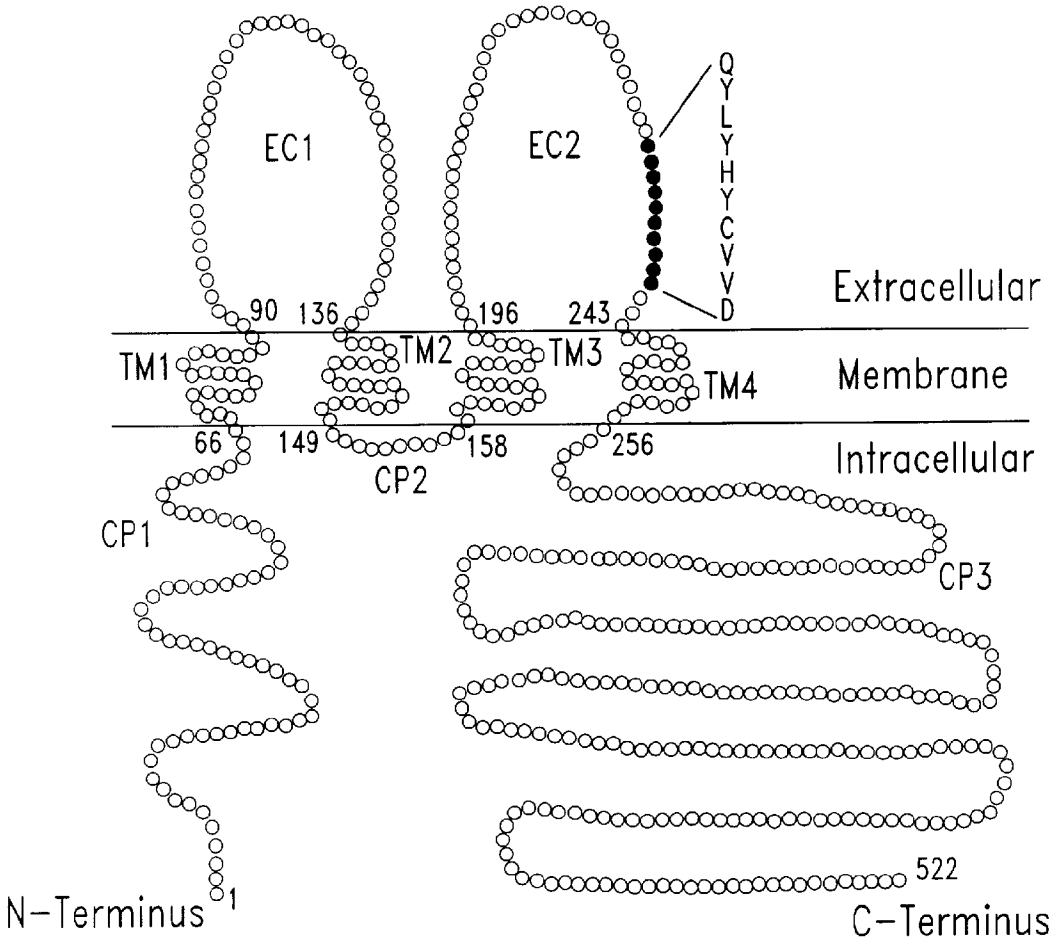
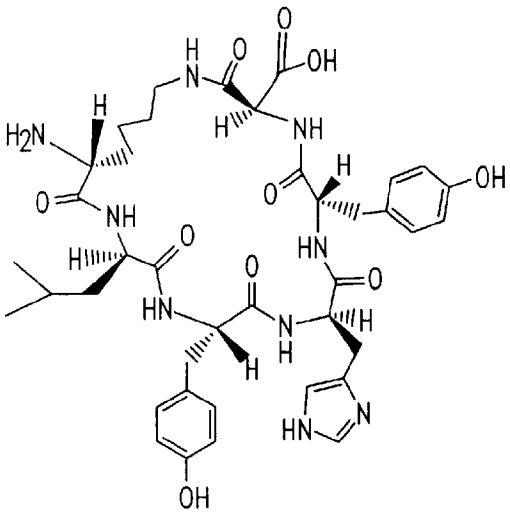

EC1
EC2
QYLYHYCVVD
Extracellular
90
136
196
243
TM1
TM2
TM3
TM4
Membrane
66
149
158
256
Intracellular
CP1
CP2
CP3
522
N-Terminus
1
C-Terminus

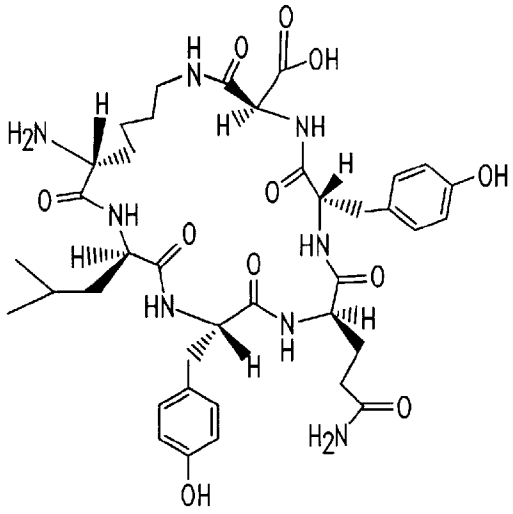
Fig. 1

```
Dog           G V N P T A Q A - - - S G S L Y S S Q I Y A M C N Q F Y A S T A T G L Y M D Q Y L Y H Y C V V D P Q E
Human         G V N P T A Q S - - - S G S L Y G S Q I Y A L C N Q F Y T P A A T G L Y V D Q Y L Y H Y C V V D P Q E
Mouse         G V N P T A Q A - - - S G S M Y G S Q I Y M I C N Q F Y T P G G T G L Y V D Q Y L Y H Y C V V D P Q E
Rat-kangaroo  G V N P R A G L G A S S G S L Y Y N Q M L M L C N Q M M S P V A G G - I M N Q Y L Y H Y C M V D P Q E
              : : : :   :           : : :   :     :         : : :               :         : : : : : : :   : : : : :
Consensus     G V N P t A q x g a s S G S l Y x s Q i y x x C N Q f y x p x a t G l y x d Q Y L Y H Y C v V D P Q E
```

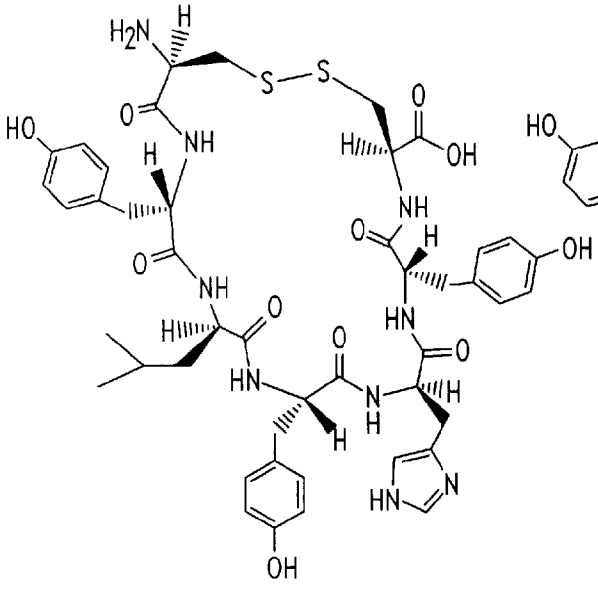
Fig. 2



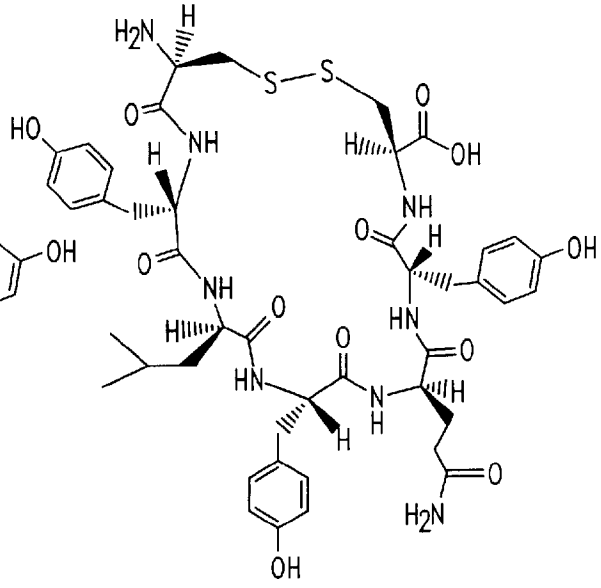
H-KLYHYD-OH



H-KLYQYD-OH

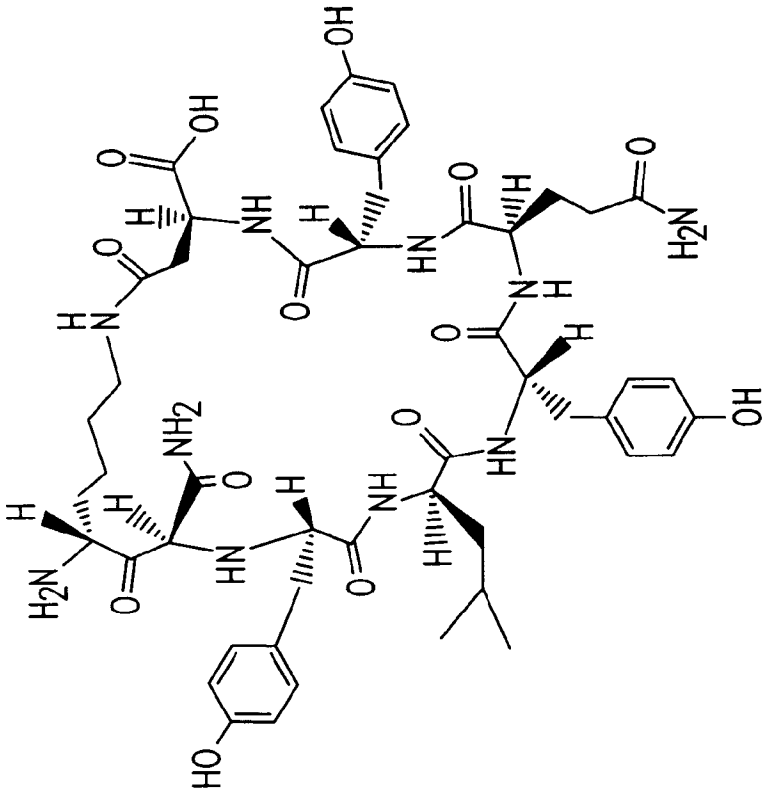


H-CYLYHYC-OH

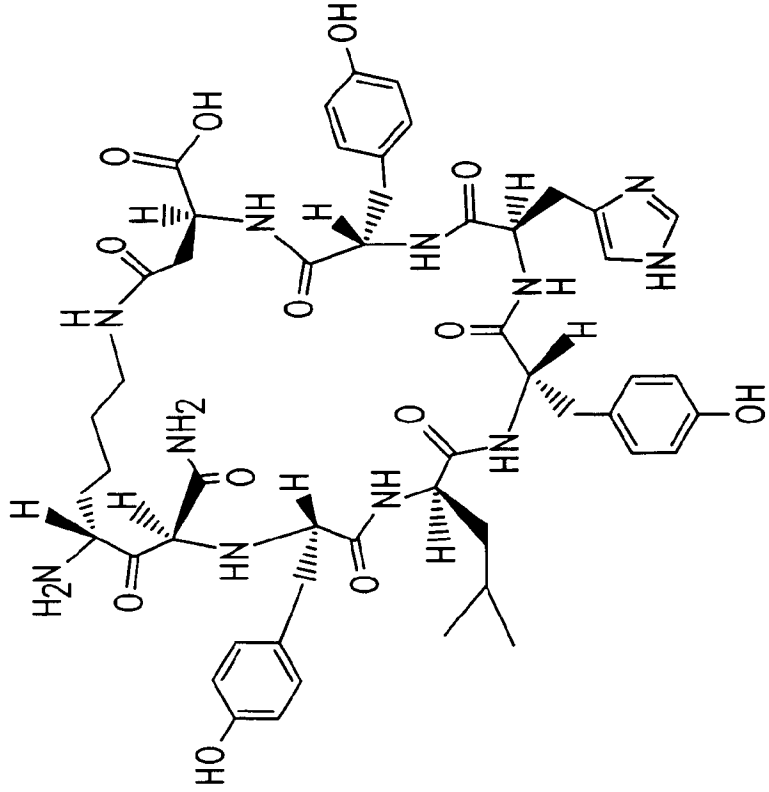


H-CYLYQYC-OH

Fig. 3A

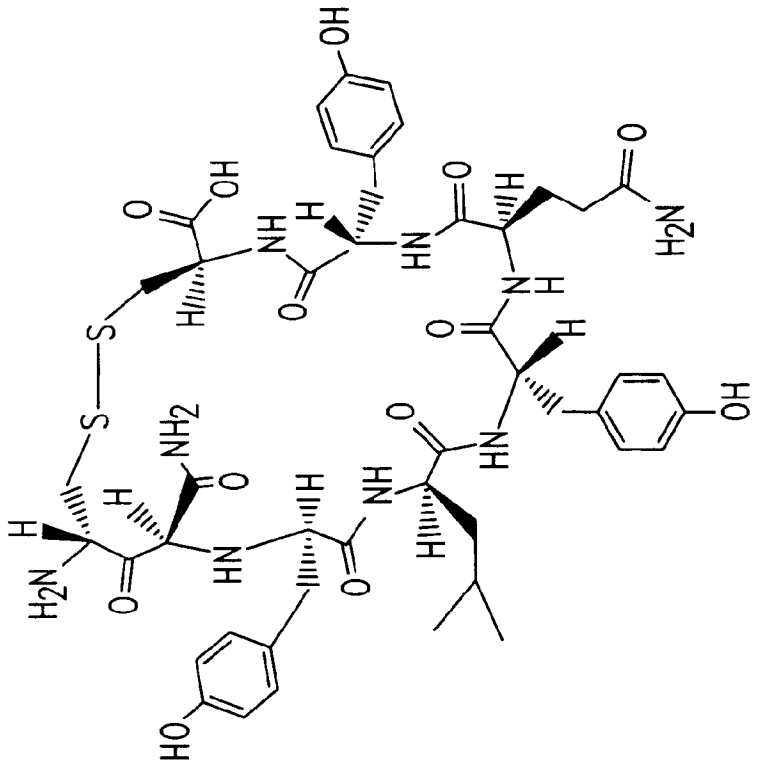


H-KQYLYQYD-OH

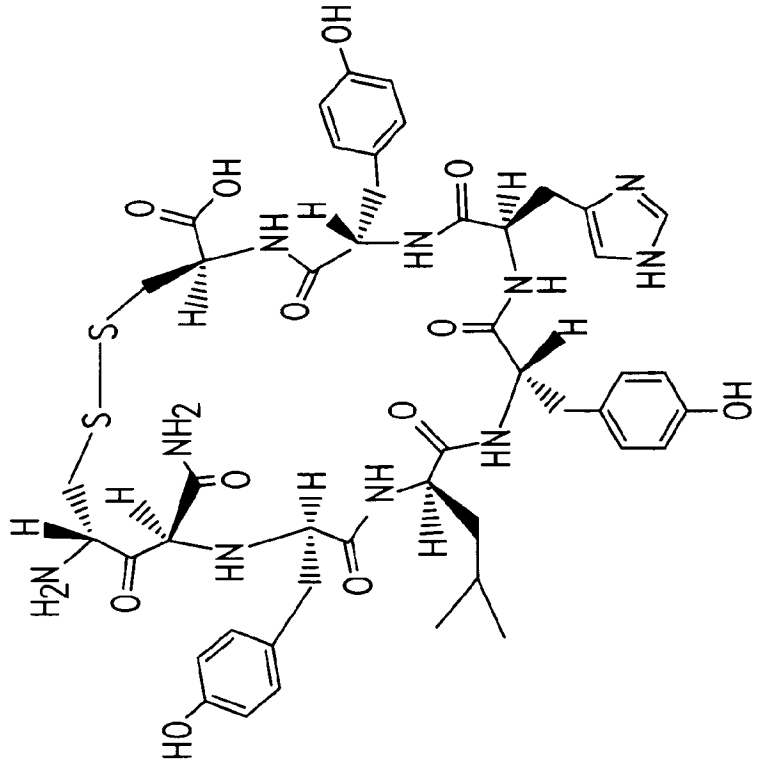


H-KQYLYHYD-OH

Fig. 3B

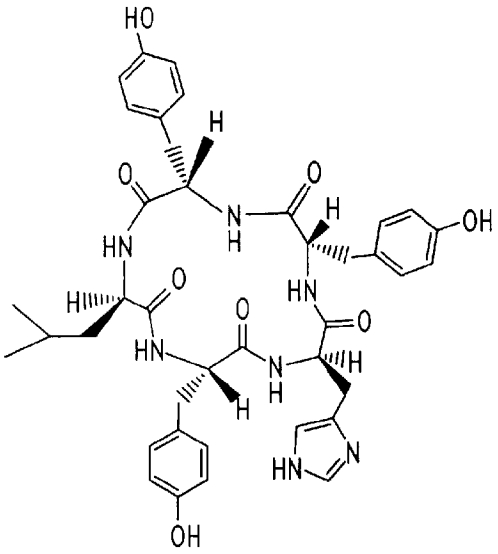


H–CQYLYQYC–OH

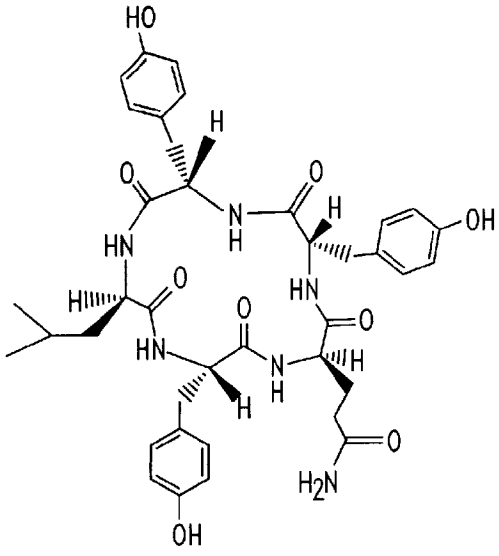


H–CQYLYHYC–OH

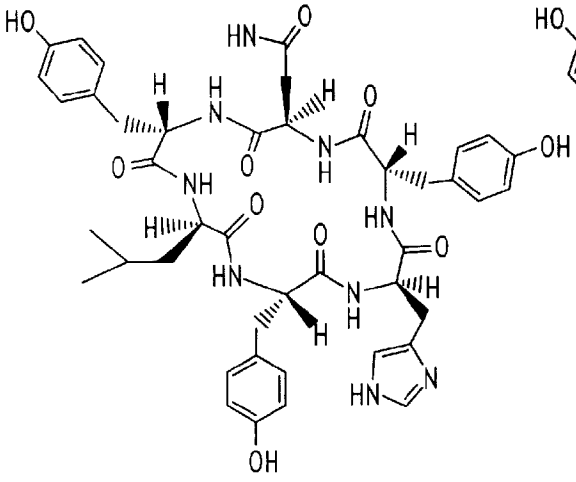
Fig. 3C



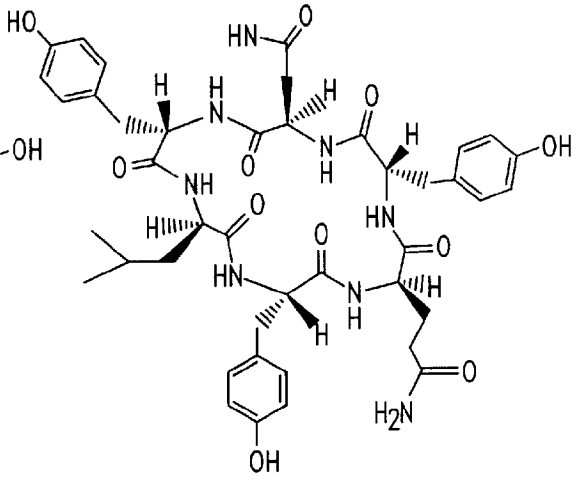
YLYHY



YLYQY



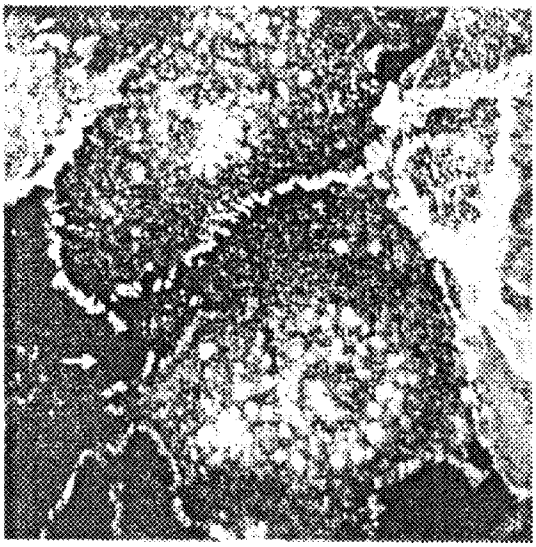
QYLYHY



QYLYHY

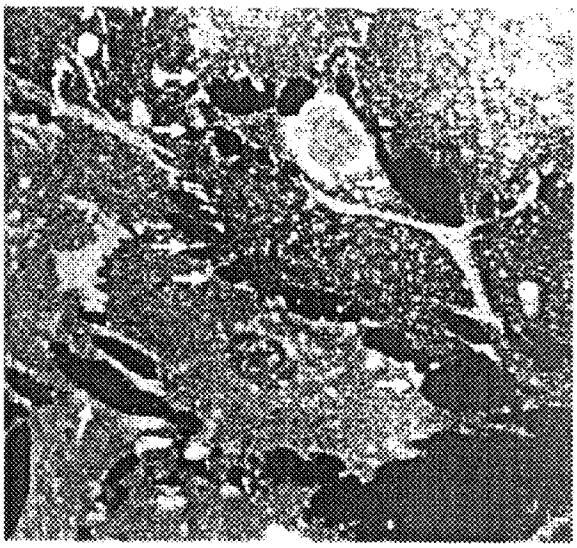
Fig. 3D

Fig. 4A



Control

Fig. 4B



Peptide 3
(100μg/mL)

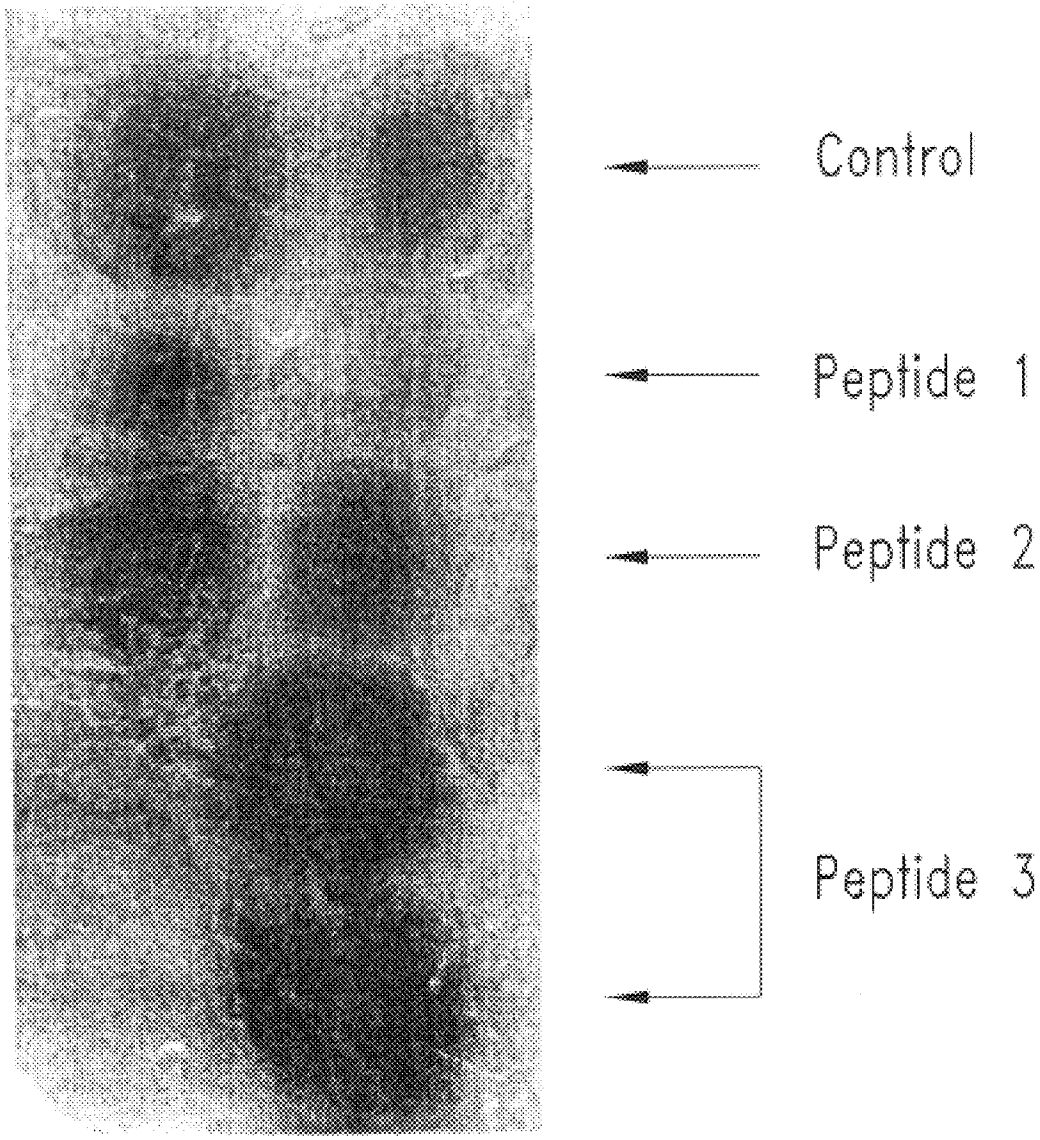


Fig. 5

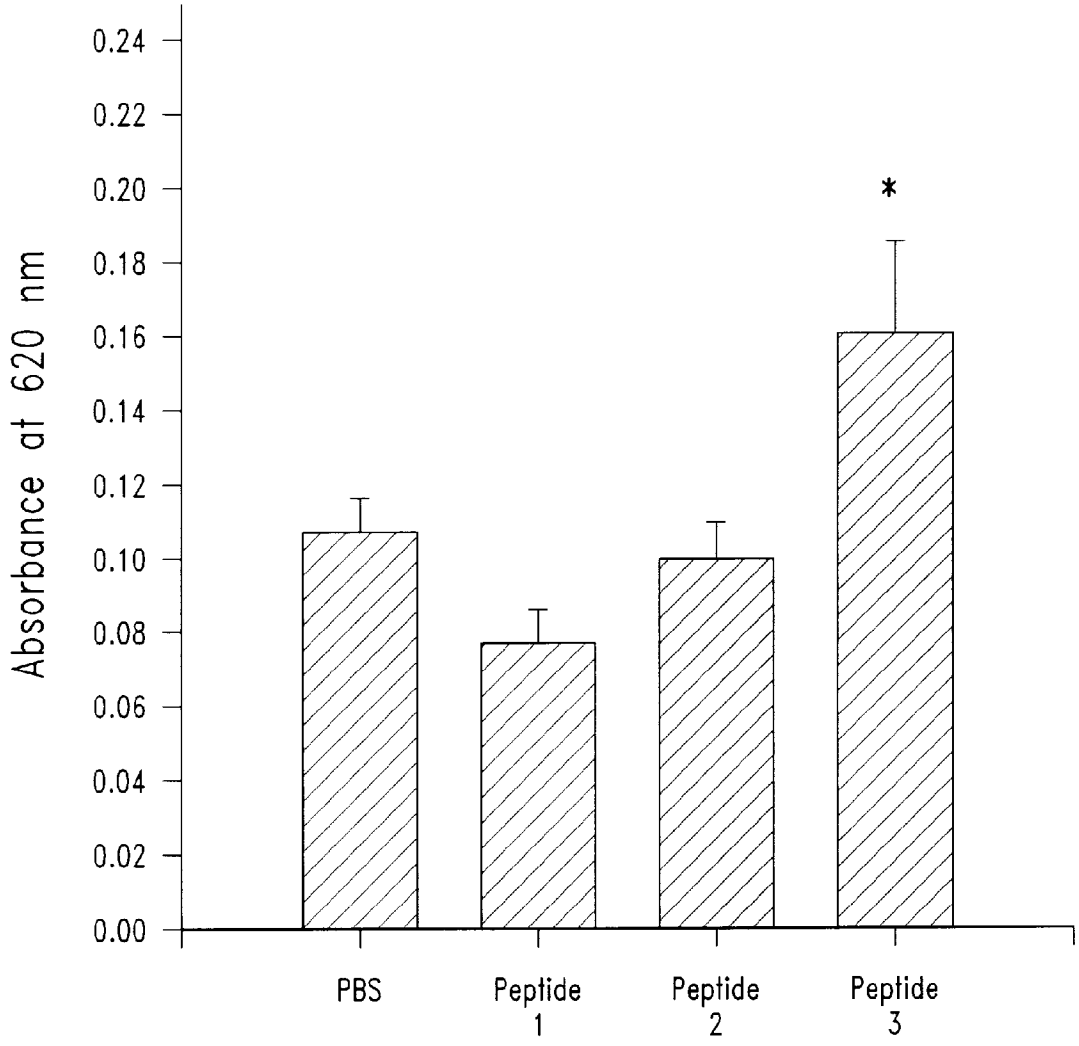

Absorbance at 620 nm
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
PBS
Peptide 1
Peptide 2
Peptide 3

Fig. 6

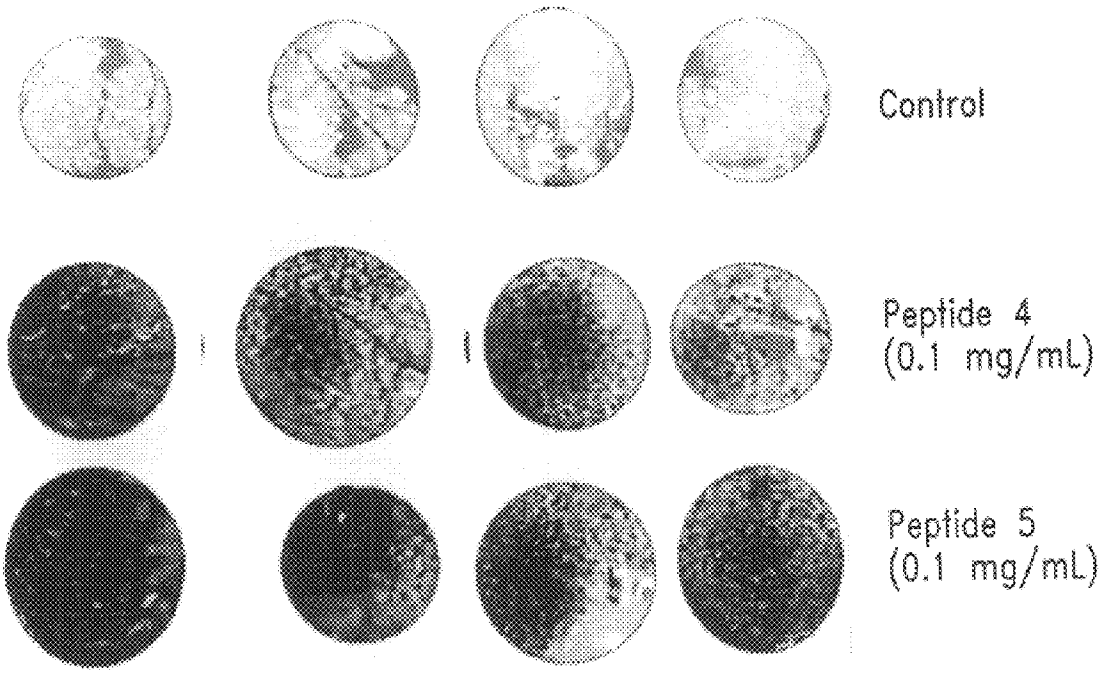


Fig. 7

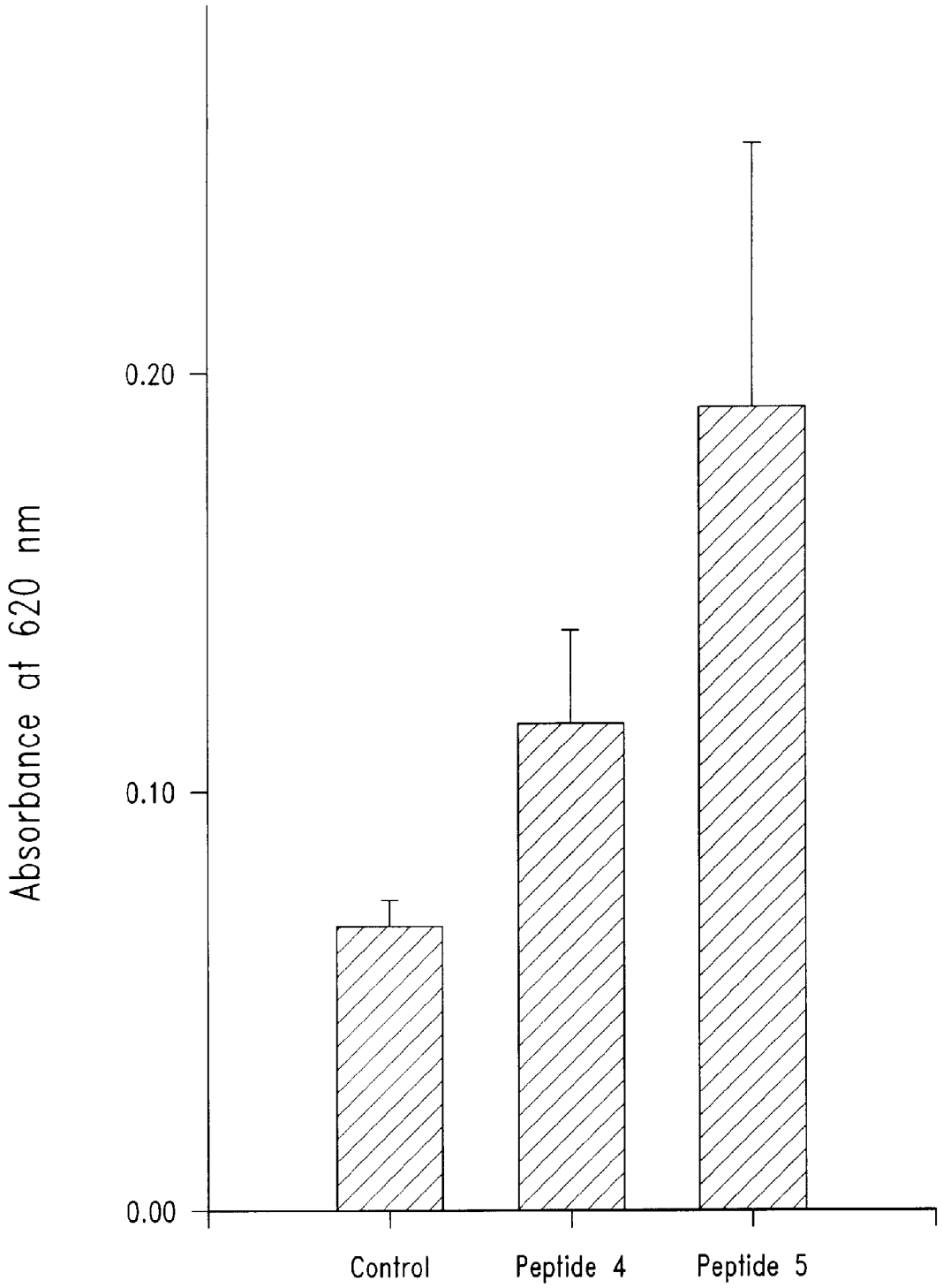


Fig. 8

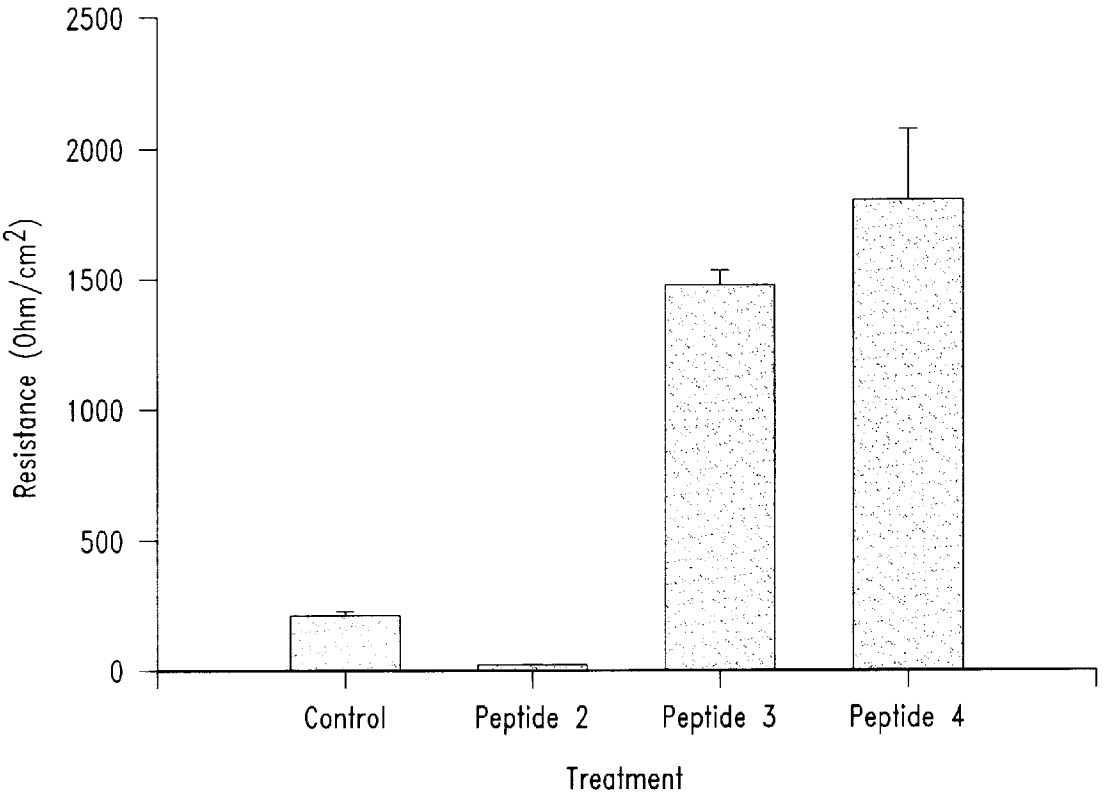

2500
2000
1500
1000
500
0
Resistance (Ohm/cm2)
Control
Peptide 2
Peptide 3
Peptide 4
Treatment

Fig. 9

COMPOUNDS AND METHODS FOR MODULATING TISSUE PERMEABILITY

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of U.S. application Ser. No. 09/001,511, filed Dec. 31, 1997.

TECHNICAL FIELD

The present invention relates generally to methods for regulating occludin-mediated processes, and more particularly to the use of modulating agents comprising an occludin cell adhesion recognition sequence and/or an antibody that specifically recognizes such a sequence for inhibiting functions such as cell adhesion and the formation of tissue permeability barriers.

BACKGROUND OF THE INVENTION

Cell adhesion is a complex process that is important for maintaining tissue integrity and generating physical and permeability barriers within the body. All tissues are divided into discrete compartments, each of which is composed of a specific cell type that adheres to similar cell types. Such adhesion triggers the formation of intercellular junctions (i.e., readily definable contact sites on the surfaces of adjacent cells that are adhering to one another), also known as tight junctions, gap junctions, spot desmosomes and belt desmosomes. The formation of such junctions gives rise to physical and permeability barriers that restrict the free passage of cells and other biological substances from one tissue compartment to another. For example, the blood vessels of all tissues are composed of endothelial cells. In order for components in the blood to enter a given tissue compartment, they must first pass from the lumen of a blood vessel through the barrier formed by the endothelial cells of that vessel. Similarly, in order for substances to enter the body via the gut, the substances must first pass through a barrier formed by the epithelial cells of that tissue. To enter the blood via the skin, both epithelial and endothelial cell layers must be crossed.

Cell adhesion is mediated by specific cell surface adhesion molecules (CAMs). There are many different families of CAMs, including the immunoglobulin, integrin, selectin and cadherin superfamilies, and each cell type expresses a unique combination of these molecules. Cadherins are a rapidly expanding family of calcium-dependent CAMs (Munro et al., *In: Cell Adhesion and Invasion in Cancer Metastasis,* P. Brodt, ed., pp. 17–34, R G Landes Co.(Austin Tex., 1996). The cadherins (abbreviated CADs) are membrane glycoproteins that generally promote cell adhesion through homophilic interactions (a CAD on the surface of one cell binds to an identical CAD on the surface of another cell). Cadherins have been shown to regulate epithelial, endothelial, neural and cancer cell adhesion, with different CADs expressed on different cell types. For example, N (neural)—cadherin is predominantly expressed by neural cells, endothelial cells and a variety of cancer cell types. E (epithelial)—cadherin is predominantly expressed by epithelial cells. VE (vascular endothelial)—cadherin is predominantly expressed by endothelial cells. Other CADs are P (placental)—cadherin, which is found in human skin, and R (retinal)—cadherin. A detailed discussion of the cadherins is provided in Munro S B et al., 1996, *In: Cell Adhesion and Invasion in Cancer Metastasis,* P. Brodt, ed., pp.17–34 (R G Landes Company, Austin Tex.) and Lampugnani and Dejana, *Curr. Opin. Cell Biol.* 9:674–682, 1997.

CAD-mediated cell adhesion triggers a cascade of events that lead to the formation of intercellular junctions, and ultimately to the establishment of permeability barriers between tissue compartments. The intercellular junction that is directly responsible for the creation of permeability barriers that prevent the diffusion of solutes through paracellular spaces is known as the tight junction, or zonula occludens (Anderson and van Itallie, *Am. J Physiol.* 269:G467–G475, 1995; Lampugnani and Dejana, *Curr. Opin. Cell Biol.* 9:674–682, 1997).

Occludin is a transmembrane component of tight junctions (Furusc et al., *J. Cell Biol.* 123:1777–1788, 1993; Furuse et al., *J. Cell Sci.* 109:429–435, 1996). This protein appears to be expressed by all endothelial cell types, as well as by most epithelial cell types. Occludin is an integral membrane protein (FIG. **1**) that is composed of two extracellular domains (EC1 and EC2), four hydrophobic domains (TM1–TM4) that transverse the plasma membrane, and three cytoplasmic domains (CP1–CP3). The structures of all known mammalian occludins are similar (FIG. **2**; Ando-Akatsuka et al., *J. Biol. Chem.* 133:43–47, 1996). Occludin is believed to be directly involved in cell adhesion and the formation of tight junctions (Furuse et al., *J. Cell Sci.* 109:429–435, 1996; Chen et al., *J. Cell Biol.* 138:891–899, 1997). It has been proposed that occludin promotes cell adhesion through homophilic interactions (an occludin on the surface of one cell binds to an identical occludin on the surface of another cell). A detailed discussion of occludin structure and function is provided by Lampugnani and Dejana, *Curr. Opin. Cell Biol.* 9:674–682, 1997.

Although cell adhesion is required for certain normal physiological functions, there are situations in which the level of cell adhesion is undesirable. For example, many pathologies (such as autoimmune diseases and inflammatory diseases) involve abnormal cellular adhesion. Cell adhesion may also play a role in graft rejection. In such circumstances, modulation of cell adhesion may be desirable.

In addition, permeability barriers arising from cell adhesion create difficulties for the delivery of drugs to specific tissues and tumors within the body. For example, skin patches are a convenient tool for administering drugs through the skin. However, the use of skin patches has been limited to small, hydrophobic molecules because of the epithelial and endothelial cell barriers. Similarly, endothelial cells render the blood capillaries largely impermeable to drugs, and the blood/brain barrier has hampered the targeting of drugs to the central nervous system. In addition, many solid tumors develop internal barriers that limit the delivery of anti-tumor drugs and antibodies to inner cells.

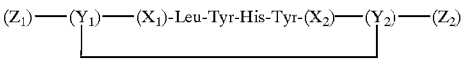
Attempts to facilitate the passage of drugs across such barriers generally rely on specific receptors or carrier proteins that transport molecules across barriers in vivo. However, such methods are often inefficient, due to low endogenous transport rates or to the poor functioning of a carrier protein with drugs. While improved efficiency has been achieved using a variety of chemical agents that disrupt cell adhesion, such agents are typically associated with undesirable side-effects, may require invasive procedures for administration and may result in irreversible effects.

Accordingly, there is a need in the art for compounds that modulate cell adhesion and improve drug delivery across permeability barriers without such disadvantages. The present invention fulfills this need and further provides other related advantages.

SUMMARY OF THE INVENTION

The present invention provides compounds and methods for modulating occludin-mediated cell adhesion and the

formation of permeability barriers. Within certain aspects, compounds provided herein comprise an occludin CAR sequence, or variant thereof that retains the ability to modulate occludin-mediated cell adhesion. Certain compounds are cyclic peptides that comprise the sequence LYHY (SEQ ID NO:1). Within certain embodiments, such cyclic peptides have the formula:



wherein X_1, and X_2 are optional, and if present, are independently selected from the group consisting of amino acid residues and combinations thereof in which the residues are linked by peptide bonds, and wherein X_1 and X_2 independently range in size from 0 to 10 residues, such that the sum of residues contained within X_1 and X_2 ranges from 1 to 12; wherein Y_1 and Y_2 are independently selected from the group consisting of amino acid residues, and wherein a covalent bond is formed between residues Y_1 and Y_2; and wherein Z_1 and Z_2 are optional, and if present, are independently selected from the group consisting of amino acid residues and combinations thereof in which the residues are linked by peptide bonds. Such cyclic peptides may comprise modifications such as an N-acetyl or N-alkoxybenzyl group and/or a C-terminal amide or ester group. Cyclic peptides may be cyclized via, for example, a disulfide bond; an amide bond between terminal functional groups, between residue side-chains or between one terminal functional group and one residue side chain; a thioether bond or $\delta_1\delta_1$-ditryptophan, or a derivative thereof.

Within other embodiments, such compounds may be linear peptides comprising the sequence LYHY (SEQ ID NO:1) or a variant thereof. Such peptides are preferably 4–30 amino acid residues in length, preferably 5–16 amino acid residues, and more preferably 6–9 amino acid residues.

Within further aspects, the present invention provides cell adhesion modulating agents that comprise a cyclic or linear peptide as described above. Within specific embodiments, such modulating agents may be linked to one or more of a targeting agent, a drug, a solid support or support molecule, or a detectable marker. Within further specific embodiments, cell adhesion modulating agents are provided that comprise a sequence selected from the group consisting of QYLYHYCVVD (SEQ ID NO:2), YLYHYCVVD (SEQ ID NO:12), LYHYCVVD (SEQ ID NO:13), QYLYHYC (SEQ ID NO:14), YLYIIYC (SEQ ID NO:15), LYIIYC (SEQ ID NO:16), QYLYHY (SEQ ID NO:17), YLYHY (SEQ ID NO:18) and derivatives of the foregoing sequences having one or more C-terminal, N-terminal and/or side chain modifications.

Within further related aspects, cell adhesion modulating agents are provided which comprise an antibody or antigen-binding fragment thereof that specifically binds to a cell adhesion recognition sequence bound by an occludin.

In addition, any of the above cell adhesion modulating agents may further comprise one or more of: (a) a cell adhesion recognition sequence that is bound by an adhesion molecule other than an occludin, wherein said cell adhesion recognition sequence is separated from any LYHY (SEQ ID NO:1) sequence(s) by a linker; and/or (b) an antibody or antigen-binding fragment thereof that specifically binds to a cell adhesion recognition sequence bound by an adhesion molecule other than an occludin.

The present invention further provides pharmaceutical compositions comprising a cell adhesion modulating agent as described above, in combination with a pharmaceutically acceptable carrier. Such compositions may further comprise a drug. In addition, or alternatively, such compositions may further comprise one or more of: (a) a peptide comprising a cell adhesion recognition sequence that is bound by an adhesion molecule other than an occludin; and/or (b) an antibody or antigen-binding fragment thereof that specifically binds to a cell adhesion recognition sequence bound by an adhesion molecule other than an occludin.

Within further aspects, methods are provided for modulating cell adhesion, comprising contacting a cadherin-expressing cell with a cell adhesion modulating agent as described above.

Within one such aspect, the present invention provides methods for increasing vasopermeability in a mammal, comprising administering to a mammal a cell adhesion modulating agent as provided above, wherein the modulating agent inhibits occludin-mediated cell adhesion.

Within another aspect, methods are provided for reducing unwanted cellular adhesion in a mammal, comprising administering to a mammal a cell adhesion modulating agent as provided above, wherein the modulating agent inhibits occludin-mediated cell adhesion.

In yet another aspect, the present invention provides methods for enhancing the delivery of a drug through the skin of a mammal, comprising contacting epithelial cells of a mammal with a cell adhesion modulating agent as provided above and a drug, wherein the modulating agent inhibits occludin-mediated cell adhesion, and wherein the step of contacting is performed under conditions and for a time sufficient to allow passage of the drug across the epithelial cells.

The present invention further provides methods for enhancing the delivery of a drug to a tumor in a mammal, comprising administering to a mammal a cell adhesion modulating agent as provided above and a drug, wherein the modulating agent inhibits occludin-mediated cell adhesion.

Within further aspects, the present invention provides methods for treating cancer in a mammal, comprising administering to a mammal a cell adhesion modulating agent as provided above, wherein the modulating agent inhibits occludin-mediated cell adhesion.

The present invention further provides methods for inhibiting angiogenesis in a mammal, comprising administering to a mammal a cell adhesion modulating agent as provided above, wherein the modulating agent inhibits occludin-mediated cell adhesion.

Within further aspects, the present invention provides methods for enhancing drug delivery to the central nervous system of a mammal, comprising administering to a mammal a cell adhesion modulating agent as provided above, wherein the modulating agent inhibits occludin-mediated cell adhesion.

Within further aspects, methods are provided for modulating the immune system of a mammal, comprising administering to a mammal a modulating agent as described above, wherein the modulating agent inhibits occludin-mediated function.

The present invention further provides methods for modulating the formation of epithelial cell tight junctions, comprising administering to a mammal a cell adhesion modulating agent that comprises the sequence LYHY (SEQ ID NO:1). Within certain embodiments, such modulating agents may stimulate the formation of epithelial cell tight junctions.

Within other aspects, the present invention provides methods for inhibiting the development of diarrhea in a patient,

comprising administering to a patient a cell adhesion modulating agent that comprises the sequence LYHY (SEQ ID NO:1), wherein the modulating agent stimulates epithelial cell adhesion.

The present invention further provides methods for enhancing wound healing in a mammal, comprising contacting a wound in a mammal with a cell adhesion modulating agent as provided above, wherein the modulating agent enhances occludin-mediated cell adhesion.

Within a related aspect, the present invention provides methods for enhancing adhesion of foreign tissue implanted within a mammal, comprising contacting a site of implantation of foreign tissue in a mammal with a cell adhesion modulating agent as provided above, wherein the modulating agent enhances occludin-mediated cell adhesion.

The present invention further provides methods for inducing apoptosis in an occludin-expressing cell, comprising contacting an occludin-expressing cell with a cell adhesion modulating agent as provided above, wherein the modulating agent inhibits occludin-mediated cell adhesion.

The present invention further provides methods for identifying an agent capable of modulating occludin-mediated cell adhesion. One such method comprises the steps of (a) culturing cells that express an occludin in the presence and absence of a candidate agent, under conditions and for a time sufficient to allow cell adhesion; and (b) visually evaluating the extent of cell adhesion among the cells.

Within another embodiment, such methods may comprise the steps of: (a) culturing normal rat kidney cells in the presence and absence of a candidate agent, under conditions and for a time sufficient to allow cell adhesion; and (b) comparing the level of cell surface occludin and E-cadherin for cells cultured in the presence of candidate agent to the level for cells cultured in the absence of candidate agent.

Within a further embodiment, such methods may comprise the steps of: (a) culturing human aortic endothelial cells in the presence and absence of a candidate agent, under conditions and for a time sufficient to allow cell adhesion; and (b) comparing the level of cell surface occludin and N-cadherin for cells cultured in the presence of candidate agent to the level for cells cultured in the absence of candidate agent.

Within yet another embodiment, such methods comprise the steps of: (a) contacting an antibody that binds to a modulating agent comprising the sequence LYHY (SEQ ID NO:1) with a test compound; and (b) detecting the level of antibody that binds to the test compound.

The present invention further provides methods for detecting the presence of occludin-expressing cells in a sample, comprising: (a) contacting a sample with an antibody that binds to an occludin comprising the sequence LYHY (SEQ ID NO:1) under conditions and for a time sufficient to allow formation of an antibody-occludin complex; and (b) detecting the level of antibody-occludin complex, and therefrom detecting the presence of occludin-expressing cells in the sample.

Within further aspects, the present invention provides kits for detecting the presence of occludin-expressing cells in a sample, comprising: (a) an antibody that binds to a modulating agent comprising the sequence LYHY (SEQ ID NO:1); and (b) a detection reagent.

The present invention further provides, within other aspects, kits for enhancing transdermal drug delivery, comprising: (a) a skin patch; and (b) a cell adhesion modulating agent, wherein said modulating agent comprises the sequence LYHY (SEQ ID NO:1), and wherein the modulating agent inhibits occludin-mediated cell adhesion.

These and other aspects of the invention will become evident upon reference to the following detailed description and attached drawings. All references disclosed herein are hereby incorporated by reference in their entirety as if each were individually noted for incorporation.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a diagram depicting the structure of a human occludin. The two extracellular domains are designated EC1 and EC2, the four hydrophobic domains that transverse the plasma membrane are represented by TM1–TM4, and the three cytoplasmic domains are denoted CP1–CP3. The occludin cell adhesion recognition sequence, LYHY (Leu-Tyr-His-Tyr; SEQ ID NO:1), along with flanking amino acid residues is shown within EC2 and is indicated by •.

FIG. **2** provides the amino acid sequences of mammalian occludin EC2 domains: human (SEQ ID NO:5), mouse (SEQ ID NO:6), dog (SEQ ID NO:7), and rat-kangaroo (SEQ ID NO:8), as indicated, along with the consensus sequence obtained using a Clustal W protein sequence alignment. The occludin cell adhesion recognition sequence, LYHY (Leu-Tyr-His-Tyr; SEQ ID NO:1), along with flanking amino acid residues is shown in bold.

FIGS. **3A–3D** provide the structures of representative cyclic peptide modulating agents.

FIGS. **4A** and **4B** are immunofluorescence photographs of monolayer cultures of human aortic endothelial cells immunolabeled for occludin (red color) and VE-cadherin (green color). Colocalization of occludin and VE-cadherin is indicated by the yellow color. Arrows indicate gaps between the cells. The cells were either not treated (FIG. **4A**), or exposed for 1 hour to 100 μg/ml H-QYLYHYCVVD-OH (SEQ ID NO:2; FIG. **4B**).

FIG. **5** is a photograph of the shaved back of a rat that received duplicate subdermal injections of either phosphate buffered saline, phosphate buffered saline containing acetyl-QYLYHYCVVD-NH_2 (SEQ ID NO:2; Peptide 1) H-QYLYHYCVVD-NH_2 (SEQ ID NO:2; Peptide 2), or H-QYLYHYCVVD-OH (SEQ ID NO:2; Peptide 3) at a concentration of 100 μg/ml, followed 15 minutes later by a single injection of Evans blue into the tail vein. The photograph was taken 15 minutes after injection of the dye.

FIG. **6** is a histogram depicting the optical densities of dimethylformamide extracts prepared from the excised injection sites shown in FIG. **5**, and showing that more dye was extracted from the sites injected with H-QYLYHYCVVD-OH (SEQ ID NO:2; Peptide 3), than from sites injected with either phosphate buffered saline, acetyl-QYLYHYCVVD-NH_2 (SEQ ID NO:2; Peptide 1) or H-QYLYHYCVVD-NH_2 (SEQ ID NO:2; Peptide 2).

FIG. **7** is a photograph of the shaved back of a rat that received duplicate subdermal injections of either phosphate buffered saline, phosphate buffered saline containing acetyl-CLYHYC-NH_2 (SEQ ID NO:3; Peptide 4), or H-CLYHYC-OH (SEQ ID NO:3; Peptide 5) at a concentration of 100 μg/ml, followed 15 minutes later by a single injection of Evans blue into the tail vein. The photograph was taken 15 minutes after injection of the dye.

FIG. **8** is a histogram depicting the optical densities of dimethylformamide extracts prepared from the excised sites of the shaved back of a rat that received duplicate subdermal injections of either phosphate buffered saline, phosphate buffered saline containing acetyl-CLYHYC-NH_2 (SEQ ID NO:3; Peptide 4), or H-CLYHYC-OH (SEQ ID NO:3; Peptide 5) at a concentration of 100 μg/ml, followed 15 minutes later by a single injection of Evans blue into the tail vein.

FIG. 9 is a histogram depicting the mean electrical resistance across MDCK cell monolayers cultured for 24 hours in medium alone (Control), or medium containing H-QYLYHYCVVD-NH_2 (Peptide 2), H-QYLYHYCVVD-COOH (Peptide 3) or N-Ac-CLYHYC-NH_2 (Peptide 4) at a concentration of 0.5 mg/ml. Duplicate measurements were taken, and error bars represent the standard deviation.

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention provides cell adhesion modulating agents comprising peptides that are capable of modulating occludin-mediated processes, such as cell adhesion. In general, to modulate occludin-mediated cell adhesion, an occludin-expressing cell is contacted with a cell adhesion modulating agent (also referred to herein as a "modulating agent") either in vivo or in vitro. It has been found, within the context of the present invention, that the second extracellular domain (EC2) of occludin contains a CAR sequence that promotes the formation of permeability barriers. Accordingly, a modulating agent may comprise at least one peptide (which may, but need not, be cyclic) that contains an occludin cell adhesion recognition (CAR) sequence and/or an antibody or fragment thereof that specifically binds to an occludin CAR sequence. In humans and certain other mammals, the CAR sequence is LYHY (Leu-Tyr-His-Tyr; SEQ ID NO:1; see FIG. 2 and SEQ ID NOs:5–8). However, the present invention further contemplates occludin CAR sequences from other organisms. Such CAR sequences may be identified based upon sequence similarity to the sequences provided herein, and the ability to modulate an occludin-mediated function may be confirmed as described herein. A modulating agent may further comprise one or more additional CAR sequences and/or antibodies (or antigen-binding fragments thereof) that specifically bind to an occludin CAR sequence. Alternatively, or in addition, a modulating agent may further comprise one or more CAR sequences for a CAM other than an occludin and/or an antibody or antigen-binding fragment thereof that specifically binds to such a CAM.

Certain modulating agents described herein inhibit cell adhesion. Such modulating agents may generally be used, for example, to treat diseases or other conditions characterized by undesirable cell adhesion or to facilitate drug delivery to a specific tissue or tumor. Within other aspects of the present invention, certain modulating agents may be used to enhance cell adhesion (e.g., to supplement or replace stitches or to facilitate wound healing). Certain modulating agents provided herein have the ability to stimulate the formation of tight junctions in epithelial cells, but not in endothelial cells. Such agents may be used, for example, for treating diarrhea.

CELL ADHESION MODULATING AGENTS

The term "cell adhesion modulating agent," as used herein, refers to a molecule comprising at least one of the following components:

(a) a linear or cyclic peptide sequence that is at least 50% identical to an occludin CAR sequence (i.e., an occludin CAR sequence or an analogue thereof that retains at least 50% sequence identity);

(b) a mimetic (e.g., peptidomimetic or small molecule mimic) of an occludin CAR sequence;

(c) a substance, such as an antibody or antigen-binding fragment thereof, that specifically binds an occludin CAR sequence; and/or

(d) a polynucleotide encoding a polypeptide that comprises an occludin CAR sequence or analogue thereof.

A modulating agent may consist entirely of one or more of the above elements, or may additionally comprise further peptide and/or non-peptide regions. Additional peptide or polynucleotide regions may be derived from occludin (preferably an extracellular domain that comprises a CAR sequence) and/or may be heterologous. Certain modulating agents comprise the occludin CAR sequence LYHY (SEQ ID NO:1) or an analogue thereof. Within certain preferred embodiments, such a modulating agent contains 4–30 consecutive amino acid residues, preferably 5–16 consecutive amino acid residues and more preferably 6–9 consecutive amino acid residues, present within an occludin.

An "occludin CAR sequence," as used herein, refers to an amino acid sequence that is present within in a naturally occurring occludin and that is capable of detectably modulating an occludin-mediated function, such as cell adhesion, as described herein. In other words, contacting an occludin-expressing cell with a peptide comprising a CAR sequence results in a detectable change in an occludin-mediated function using at least one of the representative assays provided herein. CAR sequences may be of any length, but generally comprise 4–16 amino acid residues, and preferably 5–8 amino acid residues. As noted above, the four amino acid sequence LYHY (SEQ ID NO:1) is an occludin CAR sequence.

As an alternative to comprising a native occludin CAR sequence, modulating agents as described herein may comprise an analogue or mimetic of an occludin CAR sequence. Within the specific embodiments described herein, it should be understood that an analogue or mimetic may be substituted for a native CAR sequence within any modulating agent. An analogue generally retains at least 50% identity to a native occludin CAR sequence, and modulates an occludin-mediated function as described herein. Such analogues preferably contain at least three residues of, and more preferably at least five residues of, an occludin CAR sequence. An analogue may contain any of a variety of amino acid substitutions, additions, deletions and/or modifications (e.g., side chain modifications). Preferred amino acid substitutions are conservative. A "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. Amino acid substitutions may generally be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine and valine; glycine and alanine; asparagine and glutamine; and serine, threonine, phenylalanine and tyrosine. Other groups of amino acids that may represent conservative changes include: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. The critical determining feature of an occludin CAR sequence analogue is the ability to modulate an occludin-mediated function, which may be evaluated using the representative assays provided herein.

A mimetic is a non-peptidyl compound that is conformationally similar to an occludin CAR sequence, such that it modulates an occludin-mediated function as described below. Such mimetics may be designed based on techniques

that evaluate the three dimensional structure of the peptide. For example, Nuclear Magnetic Resonance spectroscopy (NMR) and computational techniques may be used to determine the conformation of an occludin CAR sequence. NMR is widely used for structural analyses of both peptidyl and non-peptidyl compounds. Nuclear Overhauser Enhancements (NOE's), coupling constants and chemical shifts depend on the conformation of a compound. NOE data provides the interproton distance between protons through space and can be used to calculate the lowest energy conformation for the occludin CAR sequence. This information can then be used to design mimetics of the preferred conformation. Linear peptides in solution exist in many conformations. By using conformational restriction techniques it is possible to fix the peptide in the active conformation. Conformational restriction can be achieved by i) introduction of an alkyl group such as a methyl which sterically restricts free bond rotation; ii) introduction of unsaturation which fixes the relative positions of the terminal and geminal substituents; and/or iii) cyclization, which fixes the relative positions of the sidechains. Mimetics may be synthesized where one or more of the amide linkages has been replaced by isosteres, substituents or groups which have the same size or volume such as —CH_2NH—, —CSNH—, —CH_2S—, —CH=CH—, —CH_2CH_2—, —CONMe— and others. These backbone amide linkages can also be part of a ring structure (e.g., lactam). Mimetics may be designed where one or more of the side chain functionalities of the occludin CAR sequence are replaced by groups that do not necessarily have the same size or volume, but have similar chemical and/or physical properties which produce similar biological responses. Other mimetics may be small molecule mimics, which may be readily identified from small molecule libraries, based on the three-dimensional structure of the CAR sequence. It should be understood that, within embodiments described below, an analogue or mimetic may be substituted for an occludin CAR sequence.

A portion of a modulating agent that comprises an occludin CAR sequence, or analogue or mimetic thereof, may be a linear or cyclic peptide. The term "cyclic peptide," as used herein, refers to a peptide or salt thereof that comprises (1) an intramolecular covalent bond between two non-adjacent residues and (2) at least one occludin CAR sequence. The intramolecular bond may be a backbone to backbone, side-chain to backbone or side-chain to side-chain bond (i.e., terminal functional groups of a linear peptide and/or side chain functional groups of a terminal or interior residue may be linked to achieve cyclization). Preferred intramolecular bonds include, but are not limited to, disulfide, amide and thioether bonds.

In addition to one or more of the above components, a modulating agent may comprise one or more additional CAR sequences, which may or may not be occludin CAR sequences, and/or one or more antibodies or fragments thereof that specifically recognize a CAR sequence. Additional CAR sequences may be present within a cyclic peptide containing an occludin CAR sequence, within a separate cyclic peptide component of the modulating agent and/or in a non-cyclic portion of the modulating agent. Antibodies and antigen-binding fragments thereof are typically present in a non-cyclic portion of the modulating agent.

Within certain embodiments in which inhibition of cell adhesion is desired, a modulating agent may contain one occludin CAR sequence or analogue thereof. Alternatively, such an agent may comprise multiple occludin CAR sequences, which may be adjacent to one another (i.e., without intervening sequences) or in close proximity (i.e., separated by peptide and/or non-peptide linkers to give a distance between the CAR sequences that ranges from about 0.1 to 400 nm). For example, a modulating agent with adjacent LYHY sequences may comprise the peptide LYHY-LYHY (SEQ ID NO:9). A representative modulating agent with LYHY sequences in close proximity may comprise the sequence QLYHYQLYHYQLYHY (SEQ ID NO:10). One or more antibodies, or fragments thereof, may similarly be used within such embodiments, either alone or in combination with one or more CAR sequences.

In certain embodiments, a modulating agent as described above may enhance cell adhesion among epithelial cells, but not among endothelial cells. It has been found, within the context of the present invention, that certain modulating agents comprising an LYHY sequence affect endothelial and epithelial cells differently, stimulating the formation of tight junctions in epithelial cells. Such agents include H-QYLYHYCVVD-COOH (SEQ ID NO:2) and N-Ac-CLYHYC-NH_2 (SEQ ID NO:3). Terminal functional groups may influence the activity of peptide modulating agents in epithelial and endothelial cells.

Within other embodiments in which enhancement of cell adhesion is desired, a modulating agent may generally contain multiple occludin CAR sequences and/or antibodies that specifically bind to such sequences, joined by linkers as described above. Enhancement of cell adhesion may also be achieved by attachment of multiple modulating agents to a support molecule or material, as discussed further below.

A modulating agent as described herein may additionally comprise a CAR sequence for one or more different adhesion molecules (including, but not limited to, other CAMs) and/or one or more antibodies or fragments thereof that bind to such sequences. Linkers may, but need not, be used to separate such CAR sequence(s) and/or antibody sequence(s) from the LYHY sequence(s) and/or each other. Such modulating agents may generally be used within methods in which it is desirable to simultaneously disrupt cell adhesion mediated by multiple adhesion molecules. As used herein, an "adhesion molecule" is any molecule that mediates cell adhesion via a receptor on the cell's surface. Adhesion molecules include classical cadherins; atypical cadherins such as cadherin-11 (OB cadherin), cadherin-5 (VE-cadherin), cadherin-6 (K-cadherin), cadherin-7, cadherin-8, cadherin-12 (Br-cadherin, cadherin-14, cadherin-15, and PB-cadherin; other nonclassical cadherins such as desmocollins (dsc) and desmogleins (dsg); claudin; integrins; and members of the immunoglobulin supergene family, such as N-CAM and PECAM). Preferred CAR sequences for inclusion within a modulating agent include: (a) His-Ala-Val (HAV), which is bound by classical cadherins; (b) Arg-Gly-Asp (RGD), which is bound by integrins (see Cardarelli et al., *J. Biol. Chem.* 267:23159–23164, 1992); (c) KYS-FNYDGSE (SEQ ID NO:11), which is bound by N-CAM; (d) claudin CAR sequences comprising at least four consecutive amino acids present within a claudin region that has the formula: Trp-Lys/Arg-Aaa-Baa-Ser/Ala-Tyr/Phe-Caa-Gly (SEQ ID NO:47), wherein Aaa, Baa and Caa indicate independently selected amino acid residues; Lys/Arg is an amino acid that is lysine or arginine; Ser/Ala is an amino acid that is serine or alanine; and Tyr/Phe is an amino acid that is tyrosine or phenylalanine; and (e) nonclassical cadherin CAR sequences comprising at least three consecutive amino acids present within a nonclassical cadherin region that has the formula: Aaa-Phe-Baa-Ile/Leu/Val-Asp/Asn/Glu-Caa-Daa-Ser/Thr/Asn-Gly (SEQ ID NO:48), wherein Aaa, Baa, Caa and Daa are independently selected amino

acid residues; Ile/Leu/Val is an amino acid that is selected from the group consisting of isoleucine, leucine and valine, Asp/Asn/Glu is an amino acid that is selected from the group consisting of aspartate, asparagine and glutamate; and Ser/Thr/Asn is an amino acid that is selected from the group consisting of serine, threonine or asparagine. Representative claudin CAR sequences include IYSY (SEQ ID NO:49), TSSY (SEQ ID NO:50), VTAF (SEQ ID NO:51) and VSAF (SEQ ID NO:52). Representative nonclassical cadherin CAR sequences include the VE-cadherin CAR sequence DAE; the OB-cadherin CAR sequences DDK, EEY and EAQ; the dsg CAR sequences NQK, NRN and NKD and the dsc CAR sequences EKD and ERD.

A linker may be any molecule (including peptide and/or non-peptide sequences as well as single amino acids or other molecules), that does not contain a CAR sequence and that can be covalently linked to at least two peptide sequences. Using a linker, LYHY (SEQ ID NO:1)-containing peptides and other peptide or protein sequences may be joined head-to-tail (i.e., the linker may be covalently attached to the carboxyl or amino group of each peptide sequence), head-to-side chain and/or tail-to-side chain. Modulating agents comprising one or more linkers may form linear or branched structures. Within one embodiment, modulating agents having a branched structure comprise three different CAR sequences, such as RGD, LYHY (SEQ ID NO:1) and HAV. Within another embodiment, modulating agents having a branched structure may comprise, for example, LYHY (SEQ ID NO:1), along with one or more of a claudin CAR sequence; a VE-cadherin CAR sequence; a dsg CAR sequence and/or a dsc CAR sequence. Linkers preferably produce a distance between CAR sequences between 0.1 to 10,000 nm, more preferably about 0.1–400 nm. A separation distance between recognition sites may generally be determined according to the desired function of the modulating agent. For inhibitors of cell adhesion, the linker distance should be small (0.1–400 nm). For enhancers of cell adhesion, the linker distance should be 400–10,000 nm. One linker that can be used for such purposes is $(H_2N(CH_2)_nCO_2H)_m$, or derivatives thereof, where n ranges from 1 to 10 and m ranges from 1 to 4000. For example, if glycine ($H_2NCH_2CO_2H$) or a multimer thereof is used as a linker, each glycine unit corresponds to a linking distance of 2.45 angstroms, or 0.245 nm, as determined by calculation of its lowest energy conformation when linked to other amino acids using molecular modeling techniques. Similarly, aminopropanoic acid corresponds to a linking distance of 3.73 angstroms, aminobutanoic acid to 4.96 angstroms, aminopentanoic acid to 6.30 angstroms and amino hexanoic acid to 6.12 angstroms. Other linkers that may be used will be apparent to those of ordinary skill in the art and include, for example, linkers based on repeat units of 2,3-diaminopropanoic acid, lysine and/or ornithine. 2,3-Diaminopropanoic acid can provide a linking distance of either 2.51 or 3.11 angstroms depending on whether the side-chain amino or terminal amino is used in the linkage. Similarly, lysine can provide linking distances of either 2.44 or 6.95 angstroms and ornithine 2.44 or 5.61 angstroms. Peptide and non-peptide linkers may generally be incorporated into a modulating agent using any appropriate method known in the art.

The total number of CAR sequences (including occludin CAR sequence(s), with or without other CAR sequences derived from one or more adhesion molecules) present within a modulating agent may range from 1 to a large number, such as 100, preferably from 1 to 10, and more preferably from 1 to 5. Peptide modulating agents comprising multiple CAR sequences typically contain from 4 to about 1000 amino acid residues, preferably from 4 to 50 residues. When non-peptide linkers are employed, each CAR sequence of the modulating agent is present within a peptide that generally ranges in size from 4 to 50 residues in length, preferably from 4 to 25 residues, more preferably from 4 to 16 residues and still more preferably from 4 to 15 residues. Additional residue(s) that may be present on the N-terminal and/or C-terminal side of a CAR sequence may be derived from sequences that flank the LYHY sequence within naturally occurring occludins with or without amino acid substitutions and/or other modifications. Flanking sequences for mammalian occludins are shown in FIG. **2**, and in SEQ ID NOs:5–8. Alternatively, additional residues present on one or both sides of the CAR sequence(s) may be unrelated to an endogenous sequence (e.g., residues that facilitate purification or other manipulation and/or residues having a targeting or other function).

A modulating agent may contain sequences that flank the occludin CAR sequence on one or both sides, to enhance potency or specificity. A suitable flanking sequence for enhancing potency includes, but is not limited to, an endogenous sequence present in an occludin (shown in, for example, FIG. **2**).

To facilitate the preparation of modulating agents having a desired potency, nuclear magnetic resonance (NMR) and computational techniques may be used to determine the conformation of a peptide that confers a known potency. NMR is widely used for structural analysis of molecules. Cross-peak intensities in nuclear Overhauser enhancement (NOE) spectra, coupling constants and chemical shifts depend on the conformation of a compound. NOE data provide the interproton distance between protons through space. This information may be used to facilitate calculation of the lowest energy conformation for the LYHY (SEQ ID NO:1) sequence. Conformation may then be correlated with tissue specificity to permit the identification of peptides that are similarly tissue specific or have enhanced tissue specificity.

Modulating agents may be polypeptides or salts thereof, containing only amino acid residues linked by peptide bonds, or may contain non-peptide regions, such as linkers. Peptide regions of a modulating agent may comprise residues of L-amino acids, D-amino acids, or any combination thereof. Amino acids may be from natural or non-natural sources, provided that at least one amino group and at least one carboxyl group are present in the molecule; α- and β-amino acids are generally preferred. The 20 L-amino acids commonly found in proteins are identified herein by the conventional three-letter or one-letter abbreviations indicated in Table 1, and the corresponding D-amino acids are designated by a lower case one letter symbol.

TABLE 1

Amino acid one-letter and three-letter abbreviations

A	Ala	Alanine
R	Arg	Arginine
D	Asp	Aspartic acid
N	Asn	Asparagine
C	Cys	Cysteine
Q	Gln	Glutamine
E	Glu	Glutamic acid
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
L	Leu	Leucine

TABLE 1-continued

Amino acid one-letter and three-letter abbreviations		
K	Lys	Lysine
M	Met	Methionine
F	Phe	Phenylalanine
P	Pro	Proline
S	Ser	Serine
T	Thr	Threonine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
V	Val	Valine

A modulating agent may also contain rare amino acids (such as 4-hydroxyproline or hydroxylysine), organic acids or amides and/or derivatives of common amino acids, such as amino acids having the C-terminal carboxylate esterified (e.g., benzyl, methyl or ethyl ester) or amidated and/or having modifications of the N-terminal amino group (e.g., acetylation or alkoxycarbonylation), with or without any of a wide variety of side-chain modifications and/or substitutions (e.g., methylation, benzylation, t-butylation, tosylation, alkoxycarbonylation, and the like). Preferred derivatives include amino acids having a C-terminal amide group. Residues other than common amino acids that may be present with a modulating agent include, but are not limited to, 2-mercaptoaniline, 2-mercaptoproline, ornithine, diaminobutyric acid, α-aminoadipic acid, m-aminomethylbenzoic acid and α,β-diaminopropionic acid.

Certain preferred modulating agents for use within the present invention comprise at least one of the following sequences: QYLYHYCVVD (SEQ ID NO:2), YLYHYCVVD (SEQ ID NO:12), LYHYCVVD (SEQ ID NO:13), QYLYHYC (SEQ ID NO:14), YLYHYC (SEQ ID NO:15), LYHYC (SEQ ID NO:16), QYLYHY (SEQ ID NO:17), YLYHY (SEQ ID NO:18), and/or LYHY (SEQ ID NO:1), wherein each amino acid residue may, but need not, be modified as described above. Within other embodiments, a modulating agent may comprise a cyclic peptide of one of the following sequences: CLYHYC (SEQ ID NO:3), CYLYHYC (SEQ ID NO:40), CQYLYHYC(SEQ ID NO:41), KQYLYHYD (SEQ ID NO:42), YLYHY(SEQ ID NO:43), QYLYHY (SEQ ID NO:44) or KLYHYD (SEQ ID NO:45). Modulating agents comprising derivatives of any of the sequences recited herein (i.e., sequences having one or more C-terminal, N-terminal and/or side chain modifications) are also encompassed by the present invention.

Peptide modulating agents (and peptide portions of modulating agents) as described herein may be synthesized by methods well known in the art, including chemical synthesis and recombinant DNA methods. For modulating agents up to about 50 residues in length, chemical synthesis may be performed using standard solution or solid phase peptide synthesis techniques, in which a peptide linkage occurs through the direct condensation of the α-amino group of one amino acid with the α-carboxy group of the other amino acid with the elimination of a water molecule. Peptide bond synthesis by direct condensation, as formulated above, requires suppression of the reactive character of the amino group of the first and of the carboxyl group of the second amino acid. The masking substituents must permit their ready removal, without inducing breakdown of the labile peptide molecule.

In solution phase synthesis, a wide variety of coupling methods and protecting groups may be used (see Gross and Meienhofer, eds., "The Peptides: Analysis, Synthesis, Biology," Vol. 1–4 (Academic Press, 1979); Bodansky and Bodansky, "The Practice of Peptide Synthesis," 2d ed. (Springer Verlag, 1994)). In addition, intermediate purification and linear scale up are possible. Those of ordinary skill in the art will appreciate that solution synthesis requires consideration of main chain and side chain protecting groups and activation method. In addition, careful segment selection is necessary to minimize racemization during segment condensation. Solubility considerations are also a factor.

Solid phase peptide synthesis uses an insoluble polymer for support during organic synthesis. The polymer-supported peptide chain permits the use of simple washing and filtration steps instead of laborious purifications at intermediate steps. Solid-phase peptide synthesis may generally be performed according to the method of Merrifield et al., *J. Am. Chem. Soc.* 85:2149, 1963, which involves assembling a linear peptide chain on a resin support using protected amino acids. Solid phase peptide synthesis typically utilizes either the Boc or Fmoc strategy. The Boc strategy uses a 1% cross-linked polystyrene resin. The standard protecting group for α-amino functions is the tert-butyloxycarbonyl (Boc) group. This group can be removed with dilute solutions of strong acids such as 25% trifluoroacetic acid (TFA). The next Boc-amino acid is typically coupled to the amino acyl resin using dicyclohexylcarbodiimide (DCC). Following completion of the assembly, the peptide-resin is treated with anhydrous HF to cleave the benzyl ester link and liberate the free peptide. Side-chain functional groups are usually blocked during synthesis by benzyl-derived blocking groups, which are also cleaved by HF. The free peptide is then extracted from the resin with a suitable solvent, purified and characterized. Newly synthesized peptides can be purified, for example, by gel filtration, HPLC, partition chromatography and/or ion-exchange chromatography, and may be characterized by, for example, mass spectrometry or amino acid sequence analysis. In the Boc strategy, C-terminal amidated peptides can be obtained using benzhydrylamine or methylbenzhydrylamine resins, which yield peptide amides directly upon cleavage with HF.

In the procedures discussed above, the selectivity of the side-chain blocking groups and of the peptide-resin link depends upon the differences in the rate of acidolytic cleavage. Orthoganol systems have been introduced in which the side-chain blocking groups and the peptide-resin link are completely stable to the reagent used to remove the α-protecting group at each step of the synthesis. The most common of these methods involves the 9-fluorenylmethyloxycarbonyl (Fmoc) approach. Within this method, the side-chain protecting groups and the peptide-resin link are completely stable to the secondary amines used for cleaving the N-α-Fmoc group. The side-chain protection and the peptide-resin link are cleaved by mild acidolysis. The repeated contact with base makes the Merrifield resin unsuitable for Fmoc chemistry, and p-alkoxybenzyl esters linked to the resin are generally used. Deprotection and cleavage are generally accomplished using TFA.

Those of ordinary skill in the art will recognize that, in solid phase synthesis, deprotection and coupling reactions must go to completion and the side-chain blocking groups must be stable throughout the entire synthesis. In addition, solid phase synthesis is generally most suitable when peptides are to be made on a small scale.

N-acetylation of the N-terminal residue can be accomplished by reacting the final peptide with acetic anhydride before cleavage from the resin. C-amidation may be accomplished using an appropriate resin such as methylbenzhydrylamine resin using the Boc technology.

For longer modulating agents, recombinant methods are preferred for synthesis. Within such methods, all or part of a modulating agent can be synthesized in living cells, using any of a variety of expression vectors known to those of ordinary skill in the art to be appropriate for the particular host cell. Suitable host cells may include bacteria, yeast cells, mammalian cells, insect cells, plant cells, algae and other animal cells (e.g., hybridoma, CHO, myeloma). The DNA sequences expressed in this manner may encode portions of an endogenous occludin or other adhesion molecule. Such sequences may be prepared based on known cDNA or genomic sequences (see Blaschuk et al., *J. Mol. Biol.* 211:679–682, 1990), or from sequences isolated by screening an appropriate library with probes designed based on known occludin sequences. Such screens may generally be performed as described in Sambrook et al., *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., 1989 (and references cited therein). Polymerase chain reaction (PCR) may also be employed, using oligonucleotide primers in methods well known in the art, to isolate nucleic acid molecules encoding all or a portion of an endogenous adhesion molecule. To generate a nucleic acid molecule encoding a desired modulating agent, an endogenous occludin sequence may be modified using well known techniques. For example, portions encoding one or more CAR sequences may be joined, with or without separation by nucleic acid regions encoding linkers, as discussed above. Alternatively, portions of the desired nucleic acid sequences may be synthesized using well known techniques, and then ligated together to form a sequence encoding the modulating agent.

As noted above, a modulating agent may comprise one or more cyclic peptides. Such cyclic peptides may contain only one CAR sequence, or may additionally contain one or more other adhesion molecule binding sites, which may or may not be CARs. Such additional sequences may be separated by a linker (i.e., one or more peptides not derived from a CAR sequence or other adhesion molecule binding site, as described previously). Within one such embodiment, a modulating agent comprises a cyclic peptide containing two LYHY (SEQ ID NO:1) sequences. Within another embodiment, a cyclic peptide contains one LYHY (SEQ ID NO:1) and one CAR sequence recognized by a different CAM. In certain preferred embodiments, the second CAR sequence is derived from fibronectin (i.e., RGD); a classical cadherin (i.e., HAV); a claudin or a nonclassical cadherin as described above.

Cyclic peptides containing at least one occludin CAR sequence may be covalently linked to either cyclic or linear peptides containing at least one CAR sequence recognized by a different CAM, as described previously. Using a linker, cyclic LYHY-containing peptides and other cyclic or linear peptide or protein sequences may be joined head-to-tail (i.e., the linker may be covalently attached to the carboxyl or amino group of each peptide sequence), head-to-side chain and/or tail-to-side chain. Modulating agents comprising one or more linkers may form linear or branched structures. Within one embodiment, modulating agents having a branched structure comprise multiple different CAR sequences, such as various combinations of LYHY (SEQ ID NO:1), RGD, HAV, claudin CAR sequence(s) and/or nonclassical cadherin CAR sequence(s).

In addition to the CAR sequence(s), cyclic peptides generally comprise at least one additional residue, such that the size of the cyclic peptide ring ranges from 5 to about 15 residues, preferably from 5 to 10 residues. Such additional residue(s) may be present on the N-terminal and/or C-terminal side of a CAR sequence, and may be derived from sequences that flank the endogenous occludin CAR sequence with or without amino acid substitutions and/or other modifications. Alternatively, additional residues present on one or both sides of the CAR sequence(s) may be unrelated to an endogenous sequence (e.g., residues that facilitate cyclization).

Within certain preferred embodiments, as discussed below, relatively small cyclic peptides that do not contain significant sequences flanking the LYHY sequence are preferred for modulating occludin mediated cell adhesion. Such peptides may contain an N-acetyl group and a C-amide group (e.g., the 6-residue ring N-Ac-CLYHYC-NH_2 (SEQ ID NO:3). Within the context of the present invention, underlined peptide sequences indicate cyclic peptides, wherein the cyclization is performed by any suitable method as provided herein.

Within other preferred embodiments, a cyclic peptide may contain sequences that flank the LYHY (SEQ ID NO:1) sequence in a native occludin molecule on one or both sides. Such sequences may result in increased potency. Suitable flanking sequences include, but are not limited to, the endogenous sequence present in naturally occurring occludin. To facilitate the preparation of cyclic peptides having increased potency, nuclear magnetic resonance (NMR) and computational techniques may be used to determine the conformation of a peptide that confers increased potency, as described above.

Cyclic peptides as described herein may comprise residues of L-amino acids, D-amino acids, or any combination thereof. A cyclic peptide may also contain one or more rare amino acids (such as 4-hydroxyproline or hydroxylysine), organic acids or amides and/or derivatives of common amino acids, such as amino acids having the C-terminal carboxylate esterified (e.g., benzyl, methyl or ethyl ester) or amidated and/or having modifications of the N-terminal amino group (e.g., acetylation or alkoxycarbonylation), with or without any of a wide variety of side-chain modifications and/or substitutions (e.g., methylation, benzylation, t-butylation, tosylation, alkoxycarbonylation, and the like). Preferred derivatives include amino acids having an N-acetyl group (such that the amino group that represents the N-terminus of the linear peptide prior to cyclization is acetylated) and/or a C-terminal amide group (i.e., the carboxy terminus of the linear peptide prior to cyclization is amidated). Residues other than common amino acids that may be present with a cyclic peptide include, but are not limited to, penicillamine, β,β-tetramethylene cysteine, β,β-pentamethylene cysteine, β-mercaptopropionic acid, β,β-pentamethylene-β-mercaptopropionic acid, 2-mercaptobenzene, 2-mercaptoaniline, 2-mercaptoproline, ornithine, diaminobutyric acid, α-aminoadipic acid, m-aminomethylbenzoic acid and α,β-diaminopropionic acid.

Cyclic peptides as described herein may be synthesized by methods well known in the art, including recombinant

DNA methods and chemical synthesis. Following synthesis of a linear peptide (utilizing methods described herein), with or without N-acetylation and/or C-amidation, cyclization may be achieved by any of a variety of techniques well known in the art. Within one embodiment, a bond may be generated between reactive amino acid side chains. For example, a disulfide bridge may be formed from a linear peptide comprising two thiol-containing residues by oxidizing the peptide using any of a variety of methods. Within one such method, air oxidation of thiols can generate disulfide linkages over a period of several days using either basic or neutral aqueous media. The peptide is used in high dilution to minimize aggregation and intermolecular side reactions. This method suffers from the disadvantage of being slow but has the advantage of only producing H_2O as a side product. Alternatively, strong oxidizing agents such as I_2 and $K_3Fe(CN)_6$ can be used to form disulfide linkages. Those of ordinary skill in the art will recognize that care must be taken not to oxidize the sensitive side chains of Met, Tyr, Trp or His. Cyclic peptides produced by this method require purification using standard techniques, but this oxidation is applicable at acid pHs. By way of example, strong oxidizing agents can be used to perform the cyclization shown below (SEQ ID NOs: 19 and 20), in which the underlined portion is cyclized:

FmocCysAsp(t-Bu)GlyTyr(t-Bu)ProLys(Boc)Asp(t-Bu)CysLys(t-Bu)Gly-OMe→Fmoc
CysAsp(t-Bu)GlyTyr(t-Bu)ProLys(Boc)Asp(t-Bu)CysLys(t-Bu)Gly-OMe

Oxidizing agents also allow concurrent deprotection/oxidation of suitable S-protected linear precursors to avoid premature, nonspecific oxidation of free cysteine, as shown below (SEQ ID Nos:21 and 22), where X and Y=S-Trt or S-Acm:

BocCys(X)GlyAsnLeuSer(t-Bu)Thr(t-Bu)Cys(Y)MetLeuGlyOH→
BocCysGlyAsnLeuSer(t-Bu)Thr(t-Bu)CysMetLeuGlyOH

DMSO, unlike I_2 and $K_3Fe(CN)_6$, is a mild oxidizing agent which does not cause oxidative side reactions of the nucleophilic amino acids mentioned above. DMSO is miscible with H_2O at all concentrations, and oxidations can be performed at acidic to neutral pHs with harmless byproducts. Methyltrichlorosilane-diphenylsulfoxide may alternatively be used as an oxidizing agent, for concurrent deprotection/oxidation of S-Acm, S-Tacm or S-t-Bu of cysteine without affecting other nucleophilic amino acids. There are no polymeric products resulting from intermolecular disulfide bond formation. In the example below (SEQ ID NOs:23 and 24), X is Acm, Tacm or t-Bu:

H-Cys(X)TyrIleGlnAsnCys(X)ProLeuGly-NH_2→H-CysTyrIleGlnAsnCysProLeuGly-NH_2

Suitable thiol-containing residues for use in such oxidation methods include, but are not limited to, cysteine, β,β-dimethyl cysteine (penicillamine or Pen), β,β-tetramethylene cysteine (Tmc), β,β-pentamethylene cysteine (Pmc), β-mercaptopropionic acid (Mpr), β,β-pentamethylene-β-mercaptopropionic acid (Pmp), 2-mercaptobenzene, 2-mercaptoaniline and 2-mercaptoproline. Peptides containing such residues are illustrated by the following representative formulas, in which the underlined portion is cyclized, N-acetyl groups are indicated by N-Ac and C-terminal amide groups are represented by —NH_2:

i) N—Ac—Cys—Leu—Tyr—His—Tyr—Cys—NH_2 (SEQ ID NO:25)

ii) H—Cys—Leu—Tyr—His—Tyr—Cys—OH (SEQ ID NO:26)

iii) N—Ac—Cys—Gln—Tyr—Leu—Tyr—His—Tyr—Cys—NH_2 (SEQ ID NO:27)

iv) H—Cys—Gln—Tyr—Leu—Tyr—His—Tyr—Cys—OH (SEQ ID NO:28)

v) N—Ac—Cys—Tyr—Leu—Tyr—His—Tyr—Cys—NH_2 (SEQ ID NO:29)

vi) H—Cys—Tyr—Leu—Tyr—His—Tyr—Cys—OH (SEQ ID NO:30)

vii) N—Ac—Cys—Leu—Tyr—His—Tyr—Pen—NH_2 (SEQ ID NO:31)

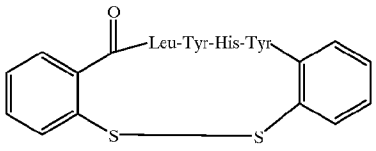
viii) N—Ac—Tmc—Leu—Tyr—His—Tyr—Cys—NH_2 (SEQ ID NO:32)

ix) N—Ac—Pmc—Leu—Tyr—His—Tyr—Cys—NH_2 (SEQ ID NO:33)

x) N—Ac—Mpr—Leu—Tyr—His—Tyr—Cys—NH_2 (SEQ ID NO:34)

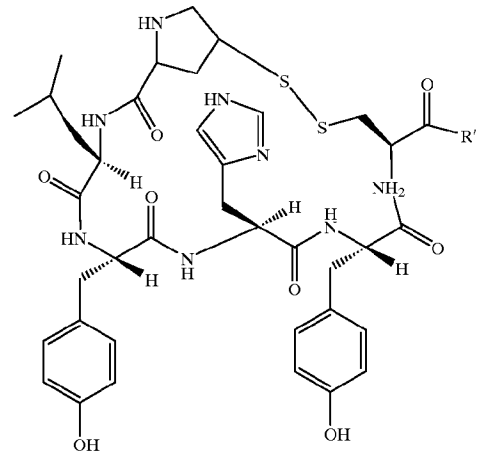
xi) N—Ac—Pmp—Leu—Tyr—His—Tyr—Cys—NH_2 (SEQ ID NO:35)

xii)



-continued

xiii)



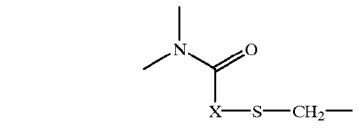
It will be readily apparent to those of ordinary skill in the art that, within each of these representative formulas, any of the above thiol-containing residues may be employed in place of one or both of the thiol-containing residues recited.

Within another embodiment, cyclization may be achieved by amide bond formation. For example, a peptide bond may be formed between terminal functional groups (i.e., the amino and carboxy termini of a linear peptide prior to cyclization). Two such cyclic peptides are YLYHY (SEQ ID NO:18) and QYLYHY (SEQ ID NO:17). Within another such embodiment, the cyclic peptide comprises a D-amino acid (e.g., yLYHY; SEQ ID NO:18). Alternatively, cyclization may be accomplished by linking one terminus and a residue side chain or using two side chains, as in KLYHYD (SEQ ID NO:36) or KQYLYHYD (SEQ ID NO:37), with or without an N-terminal acetyl group and/or a C-terminal amide. Residues capable of forming a lactam bond include lysine, ornithine (Orn), α-amino adipic acid, m-aminomethylbenzoic acid, α,β-diaminopropionic acid, glutamate or aspartate.

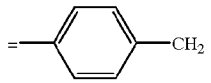
Methods for forming amide bonds are well known in the art and are based on well established principles of chemical reactivity. Within one such method, carbodiimide-mediated lactam formation can be accomplished by reaction of the carboxylic acid with DCC, DIC, EDAC or DCCI, resulting in the formation of an O-acylurea that can be reacted immediately with the free amino group to complete the cyclization. The formation of the inactive N-acylurea, resulting from O→N migration, can be circumvented by converting the O-acylurea to an active ester by reaction with an N-hydroxy compound such as 1-hydroxybenzotriazole, 1-hydroxysuccinimide, 1-hydroxynorbornene carboxamide or ethyl 2-hydroximino-2-cyanoacetate. In addition to minimizing O→N migration, these additives also serve as catalysts during cyclization and assist in lowering racemization. Alternatively, cyclization can be performed using the azide method, in which a reactive azide intermediate is generated from an alkyl ester via a hydrazide. Hydrazinolysis of the terminal ester necessitates the use of a t-butyl group for the protection of side chain carboxyl functions in the acylating component. This limitation can be overcome by using diphenylphosphoryl acid (DPPA), which furnishes an azide directly upon reaction with a carboxyl group. The slow reactivity of azides and the formation of isocyanates by their disproportionation restrict the usefulness of this method. The mixed anhydride method of lactam formation is widely used because of the facile removal of reaction by-products. The anhydride is formed upon reaction of the carboxylate anion with an alkyl chloroformate or pivaloyl chloride. The attack of the amino component is then guided to the carbonyl carbon of the acylating component by the electron donating effect of the alkoxy group or by the steric bulk of the pivaloyl chloride t-butyl group, which obstructs attack on the wrong carbonyl group. Mixed anhydrides with phosphoric acid derivatives have also been successfully used. Alternatively, cyclization can be accomplished using activated esters. The presence of electron withdrawing substituents on the alkoxy carbon of esters increases their susceptibility to aminolysis. The high reactivity of esters of p-nitrophenol, N-hydroxy compounds and polyhalogenated phenols has made these "active esters" useful in the synthesis of amide bonds. The last few years have witnessed the development of benzotriazolyloxytris-(dimethylamino) phosphonium hexafluorophosphonate (BOP) and its congeners as advantageous coupling reagents. Their performance is generally superior to that of the well established carbodiimide amide bond formation reactions.

Within a further embodiment, a thioether linkage may be formed between the side chain of a thiol-containing residue and an appropriately derivatized α-amino acid. By way of example, a lysine side chain can be coupled to bromoacetic acid through the carbodiimide coupling method (DCC, EDAC) and then reacted with the side chain of any of the thiol containing residues mentioned above to form a thioether linkage. In order to form dithioethers, any two thiol containing side-chains can be reacted with dibromoethane and diisopropylamine in DMF. Examples of thiol-containing linkages are shown below:

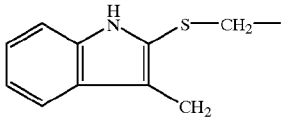
i.



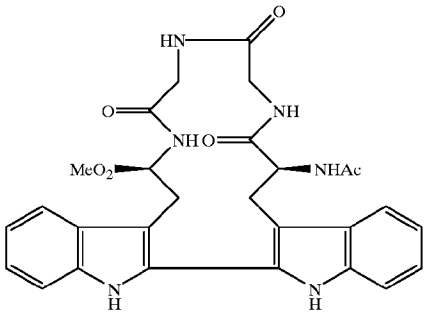
X = $(CH_2)_4$
= CH_2



ii.



Cyclization may also be achieved using δ_1,δ_1-Ditryptophan (i.e., Ac-Trp-Gly-Gly-Trp-OMe) (SEQ ID NO:38), as shown below:



Representative structures of cyclic peptides are provided in FIG. **3**. Within FIG. **3**, certain cyclic peptides having the ability to modulate cell adhesion (shown on the left) are paired with similar inactive structures (on the right). The structures and formulas recited herein are provided solely for the purpose of illustration, and are not intended to limit the scope of the cyclic peptides described herein.

As noted above, instead of (or in addition to) an occludin CAR sequence, a modulating agent may comprise an antibody, or antigen-binding fragment thereof, that specifically binds to a occludin CAR sequence. As used herein, an antibody, or antigen-binding fragment thereof, is said to "specifically bind" to a occludin CAR sequence (with or without flanking amino acids) if it reacts at a detectable level with a peptide containing that sequence, and does not react detectably with peptides containing a different CAR sequence or a sequence in which the order of amino acid residues in the occludin CAR sequence and/or flanking sequence is altered. Such antibody binding properties may be assessed using an ELISA, as described by Newton et al., *Develop. Dynamics* 197:1–13, 1993.

Polyclonal and monoclonal antibodies may be raised against an occludin CAR sequence using conventional techniques. See, e.g., Harlow and Lane, *Antibodies: A Laboratory Manual,* Cold Spring Harbor Laboratory, 1988. In one such technique, an immunogen comprising the occludin CAR sequence is initially injected into any of a wide variety of mammals (e.g., mice, rats, rabbits, sheep or goats). The smaller immunogens (i.e., less than about 20 amino acids) should be joined to a carrier protein, such as bovine serum albumin or keyhole limpet hemocyanin. Following one or more injections, the animals are bled periodically. Polyclonal antibodies specific for the CAR sequence may then be purified from such antisera by, for example, affinity chromatography using the modulating agent or antigenic portion thereof coupled to a suitable solid support.

Monoclonal antibodies specific for the occludin CAR sequence may be prepared, for example, using the technique of Kohler and Milstein, *Eur. J. Immunol.* 6:511–519, 1976, and improvements thereto. Briefly, these methods involve the preparation of immortal cell lines capable of producing antibodies having the desired specificity from spleen cells obtained from an animal immunized as described above. The spleen cells are immortalized by, for example, fusion with a myeloma cell fusion partner, preferably one that is syngeneic with the immunized animal. Single colonies are selected and their culture supernatants tested for binding activity against the modulating agent or antigenic portion thereof. Hybridomas having high reactivity and specificity are preferred.

Monoclonal antibodies may be isolated from the supernatants of growing hybridoma colonies, with or without the use of various techniques known in the art to enhance the yield. Contaminants may be removed from the antibodies by conventional techniques, such as chromatography, gel filtration, precipitation, and extraction. Antibodies having the desired activity may generally be identified using immunofluorescence analyses of tissue sections, cell or other samples where the target occludin is localized.

Within certain embodiments, the use of antigen-binding fragments of antibodies may be preferred. Such fragments include Fab fragments, which may be prepared using standard techniques. Briefly, immunoglobulins may be purified from rabbit serum by affinity chromatography on Protein A bead columns (Harlow and Lane, *Antibodies: A Laboratory Manual,* Cold Spring Harbor Laboratory, 1988; see especially page 309) and digested by papain to yield Fab and Fc fragments. The Fab and Fc fragments may be separated by affinity chromatography on protein A bead columns (Harlow and Lane, 1988, pages 628–29).

EVALUATION OF MODULATING AGENT ACTIVITY

As noted above, modulating agents as described herein are capable of modulating occludin-mediated cell adhesion. The ability of an agent to modulate cell adhesion may generally be evaluated in vitro by assaying the effect on endothelial and/or epithelial cell adhesion using, for example, any of a variety of immunostaining protocols and/or plating assays. In general, a modulating agent is an inhibitor of cell adhesion if contact of the test cells with the modulating agent results in a discernible disruption of cell adhesion using one or more representative assays provided herein. Modulating agents that enhance cell adhesion (e.g. agents comprising multiple LYHY (SEQ ID NO:1) sequences and/or linked to a support molecule or material) are considered to be modulators of cell adhesion if they are capable of promoting cell adhesion, as judged by plating assays to assess either endothelial or epithelial cell adhesion to a modulating agent attached to a support material, such as tissue culture plastic.

The ability of an agent to modulate cell adhesion may generally be evaluated in vivo by assessing the effect on vascular permeability utilizing the Miles assay (McClure et al., *J. Pharmacological & Toxicological Methods* 32:49–52, 1994). Briefly, a candidate modulating agent may be dissolved in phosphate buffered saline (PBS) at a concentration of 100 μg/ml. Adult rats may be given 100 μl subdermal

injections of each peptide solution into their shaved backs, followed 15 minutes later by a single 250 μl injection of 1% Evans blue dissolved in PBS into their tail veins. The subdermal injection sites may be visually monitored for the appearance of blue dye. Once the dye appears (about 15 minutes after injection), each subdermal injection site may be excised, weighed, and placed in 1 ml dimethylformamide for 24 hours to extract the dye. The optical density of the dye extracts may then be determined at 620 nm. In general, the injection of 0.1 ml of modulating agent (at a concentration of 0.1 mg/ml) into the backs of rats causes an increase of dye accumulation at the injection sites of at least 50%, as compared to dye accumulation at sites into which PBS has been injected.

The effect of a modulating agent on endothelial cell adhesion may generally be evaluated using immunolocalization techniques. Human aortic endothelial cells (HAEC) may be cultured on fibronectin-coated coverslips (fibronectin may be obtained from Sigma, St. Louis, Mo.) according to the procedures of Jaffe et al., *J. Clin. Invest.* 52:2745–2756, 1973. Briefly, human endothelial cells may be maintained in EGM (endothelial cell growth medium; Clonetics, San Diego, Calif.) and used for experiments at passage 4. Confluent cultures of HAEC may be exposed to either a candidate modulating agent (final concentration 100 μg/ml EGM), or EGM alone for 1 hour. The cells are then be fixed for 30 minutes at 4° C. in 95% ethanol, followed by fixation in acetone for 1 minute at 4° C. (Furuse et al., *J. Cell Biol.* 123:1777–1788, 1993). After fixation, the cells may be probed with either mouse anti-VE-cadherin antibodies (Hemeris, Sassenage, France; diluted 1:250 in 0.1% dried skim milk powder dissolved in PBS), or rabbit anti-occludin antibodies (Zymed, South San Francisco, Calif.; diluted 1:300 in 0.1% dried skim milk powder dissolved in PBS) for 1 hour at 37° C. The cells may then be washed with 0.1% dried skim milk powder dissolved in PBS (three washes, 5 minutes/wash), and probed with secondary antibodies (donkey anti-mouse Cy3, or donkey anti-rabbit Cy5 diluted 1:250 in 0.1% dried skim milk powder dissolved in PBS; Jackson Immunoresearch Laboratories Inc., Westgrove, Pa.) for 1 hour at 37° C. The cells may then be washed again with in 0.1% dried skim milk powder dissolved in PBS and mounted in a solution composed of 50% glycerol and 50% PBS to which phenylenediamine (Sigma, St. Louis, Mo.) has been added to a final concentration of 1 mg/ml. The sample may then be analyzed using a Bio-Rad MRC 1000 confocal microscope with Laser Sharp software version 2.1T (Bio-Rad, Hercules, Calif.). In general, 0.1 mg/ml of modulating agent results in the appearance of intercellular gaps within the monolayer cultures and a decrease of at least 50% in the surface expression of occludin and VE-cadherin, as compared to monolayer cultures that were not exposed to the modulating agent.

Within certain cell adhesion assays, the addition of a modulating agent to cells that express occludin results in disruption of cell adhesion. An "occludin-expressing cell," as used herein, may be any type of cell that expresses occludin on the cell surface at a detectable level, using standard techniques such as immunocytochemical protocols (e.g., Blaschuk and Farookhi, *Dev. Biol.* 136:564–567, 1989). Occludin-expressing cells include endothelial, epithelial and/or cancer cells. For example, such cells may be plated under standard conditions that, in the absence of modulating agent, permit cell adhesion. In the presence of modulating agent (e.g., 100 μg/mL), disruption of cell adhesion may be determined visually within 24 hours, by observing retraction of the cells from one another.

Within another such assay, the effect of a modulating agent on normal at kidney (NRK) cells may be evaluated. According to a representative procedure, NRK cells (ATCC #1571-CRL) may be plated at 10–20,000 cells per 35 mm tissue culture flasks containing DMEM with 10% FCS and sub-cultured periodically (Laird et al., *J. Cell Biol.* 131:1193–1203, 1995). Cells may be harvested and replated in 35 mm tissue culture flasks containing 1 mm coverslips and incubated until 50–65% confluent (24–36 hours). At this time, coverslips may be transferred to a 24-well plate, washed once with fresh DMEM and exposed to modulating agent at a concentration of, for example, 0.1 mg/mL for 24 hours. Fresh modulating agent may then be added, and the cells left for an additional 24 hours. Cells may be fixed with 100% methanol for 10 minutes and then washed three times with PBS. Coverslips may be blocked for 1 hour in 2% BSA/PBS and incubated for a further 1 hour in the presence of rabbit anti-occludin antibody ((Zymed, South San Francisco, Calif.) and mouse anti-E-cadherin antibody (Transduction Labs, 1:250 dilution). Primary and secondary antibodies may be diluted in 2% BSA/PBS. Following incubation in the primary antibody, coverslips may be washed three times for 5 minutes each in PBS and incubated for 1 hour with donkey anti-mouse Cy3 and donkey anti-rabbit Cy5 (Jackson Immunoresearch Laboratories Inc., Westgrove, Pa.) for 1 hour at 37° C. Following further washes in PBS (3×5 min) coverslips can be mounted and viewed by confocal microscopy.

In the absence of modulating agent, NRK cells form characteristic tightly adherent monolayers with a cobblestone morphology in which cells display a polygonal shape. NRK cells that are treated with a modulating agent that disrupts occludin-mediated cell adhesion may assume a non-polygonal and elongated morphology (i.e., a fibroblast-like shape) within 48 hours of treatment with 0.1 mg/mL of modulating agent. Gaps appear in confluent cultures of such cells. In addition, 0.1 mg/mL of such a modulating agent reproducibly induces a readily apparent reduction in cell surface staining of occludin and E-cadherin, as judged by immunofluorescence microscopy (Laird et al., *J. Cell Biol.* 131:1193–1203, 1995), of at least 75% within 48 hours.

A third cell adhesion assay involves evaluating the effect of a modulating agent on permeability of adherent endothelial cell monolayers. The effects of a modulating agent on the permeability of endothelial cell monolayers may be assessed utilizing the protocols of Ehringer et al., *J. Cell. Physiol.* 167:562–569, 1996. HAEC can be seeded onto inserts in 24-well plates (Becton-Dickenson, Franklin Lake, N.J.) and cultured in EGM. Confluent cell monolayers may be exposed to either modulating agent (final concentration 100 μg/ml EGM), or EGM alone for 1 hour. The inserts may then be transferred to 24-chamber plates (Becton-Dickenson) for permeability assays. Perfusate (0.5% bovine serum albumin, fraction V (Sigma) dissolved in 15 mM HEPES, pH 7.4) and FITC-Dextran (50 μg/ml HEPES buffer; MW 12 kDa; Sigma) may be added to each well (1 ml/well and 50 μl/well, respectively), and the cells incubated at 37° C. for 30 min. Aliquots of 100 μl may then be removed from the lower chamber and the optical density of the solution determined at a wavelength of 450 nm. In general, the presence of 100 μg/mL modulating agent that enhances the permeability of endothelial cell monolayers results in a statistically significant increase in the amount of marker in the receptor compartment after 1 hour.

Yet another assay evaluates the effect of an occludin modulating agent on the electrical resistance across a monolayer of cells. For example, Madin Darby canine kidney

(MDCK) cells can be exposed to the modulating agent dissolved in medium (e.g., at a final concentration of 0.5 mg/ml for a period of 24 hours). The effect on electrical resistance can be measured using standard techniques. This assay evaluates the effect of a modulating agent on tight junction formation in epithelial cells. In general, the presence of 500 μg/mL modulating agent should result in a statistically significant increase or decrease in electrical resistance after 24 hours.

MODULATING AGENT MODIFICATION AND FORMULATIONS

A modulating agent as described herein may, but need not, be linked to one or more additional molecules. In particular, as discussed below, it may be beneficial for certain applications to link multiple modulating agents (which may, but need not, be identical) to a support material, such as a single molecule (e.g., keyhole limpet hemocyanin) or a solid support, such as a polymeric matrix (which may be formulated as a membrane or microstructure, such as an ultra thin film), a container surface (e.g., the surface of a tissue culture plate or the interior surface of a bioreactor), or a bead or other particle, which may be prepared from a variety of materials including glass, plastic or ceramics. For certain applications, biodegradable support materials are preferred, such as cellulose and derivatives thereof, collagen, spider silk or any of a variety of polyesters (e.g., those derived from hydroxy acids and/or lactones) or sutures (see U.S. Pat. No. 5,245,012). Within certain embodiments, modulating agents and molecules comprising other CAR sequence(s) (e.g., an HAV sequence) may be attached to a support such as a polymeric matrix, preferably in an alternating pattern.

Suitable methods for linking a modulating agent to a support material will depend upon the composition of the support and the intended use, and will be readily apparent to those of ordinary skill in the art. Attachment may generally be achieved through noncovalent association, such as adsorption or affinity or, preferably, via covalent attachment (which may be a direct linkage between a modulating agent and functional groups on the support, or may be a linkage by way of a cross-linking agent). Attachment of a modulating agent by adsorption may be achieved by contact, in a suitable buffer, with a solid support for a suitable amount of time. The contact time varies with temperature, but is generally between about 5 seconds and 1 day, and typically between about 10 seconds and 1 hour.

Covalent attachment of a modulating agent to a molecule or solid support may generally be achieved by first reacting the support material with a bifunctional reagent that will also react with a functional group, such as a hydroxyl, thiol, carboxyl, ketone or amino group, on the modulating agent. For example, a modulating agent may be bound to an appropriate polymeric support or coating using benzoquinone, by condensation of an aldehyde group on the support with an amine and an active hydrogen on the modulating agent or by condensation of an amino group on the support with a carboxylic acid on the modulating agent. A preferred method of generating a linkage is via amino groups using glutaraldehyde. A modulating agent may be linked to cellulose via ester linkages. Similarly, amide linkages may be suitable for linkage to other molecules such as keyhole limpet hemocyanin or other support materials. Multiple modulating agents and/or molecules comprising other CAR sequences may be attached, for example, by random coupling, in which equimolar amounts of such molecules are mixed with a matrix support and allowed to couple at random.

Although modulating agents as described herein may preferentially bind to specific tissues or cells, and thus may be sufficient to target a desired site in vivo, it may be beneficial for certain applications to include an additional targeting agent. Accordingly, a targeting agent may also, or alternatively, be linked to a modulating agent to facilitate targeting to one or more specific tissues. As used herein, a "targeting agent," may be any substance (such as a compound or cell) that, when linked to a modulating agent enhances the transport of the modulating agent to a target tissue, thereby increasing the local concentration of the modulating agent. Targeting agents include antibodies or fragments thereof, receptors, ligands and other molecules that bind to cells of, or in the vicinity of, the target tissue. Known targeting agents include serum hormones, antibodies against cell surface antigens, lectins, adhesion molecules, tumor cell surface binding ligands, steroids, cholesterol, lymphokines, fibrinolytic enzymes and those drugs and proteins that bind to a desired target site. Among the many monoclonal antibodies that may serve as targeting agents are anti-TAC, or other interleukin-2 receptor antibodies; 9.2.27 and NR-ML-05, reactive with the 250 kilodalton human melanoma-associated proteoglycan; and NR-LU-10, reactive with a pancarcinoma glycoprotein. An antibody targeting agent may be an intact (whole) molecule, a fragment thereof, or a functional equivalent thereof. Examples of antibody fragments are F(ab')2, -Fab', Fab and F[v] fragments, which may be produced by conventional methods or by genetic or protein engineering. Linkage is generally covalent and may be achieved by, for example, direct condensation or other reactions, or by way of bi- or multifunctional linkers.

For certain embodiments, it may be beneficial to also, or alternatively, link a drug to a modulating agent. As used herein, the term "drug" refers to any bioactive agent intended for administration to a mammal to prevent or treat a disease or other undesirable condition. Drugs include hormones, growth factors, proteins, peptides and other compounds. The use of certain specific drugs within the context of the present invention is discussed below.

Modulating agents as described herein may be present within a pharmaceutical composition. A pharmaceutical composition comprises one or more modulating agents in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may comprise buffers (e.g., neutral buffered saline or phosphate buffered saline), carbohydrates (e.g., glucose, mannose, sucrose or dextrans), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, chelating agents such as EDTA or glutathione, adjuvants (e.g., aluminum hydroxide) and/or preservatives. Within yet other embodiments, compositions of the present invention may be formulated as a lyophilizate. One or more modulating agents (alone or in combination with a targeting agent and/or drug) may, but need not, be encapsulated within liposomes using well known technology. Compositions of the present invention may be formulated for any appropriate manner of administration, including for example, topical, oral, nasal, intravenous, intracranial, intraperitoneal, subcutaneous, or intramuscular administration.

For certain embodiments, as discussed below, a pharmaceutical composition may further comprise a modulator of cell adhesion that is mediated by one or more molecules other than occludin. Such modulators may generally be prepared as described above, incorporating one or more non-occludin CAR sequences and/or antibodies thereto in place of the occludin CAR sequence and antibodies. Such

compositions are particularly useful for situations in which it is desirable to inhibit cell adhesion mediated by multiple cell adhesion molecules, such as cadherins (e.g., classical cadherins, E-cadherin, Dsg and Dsc); integrins; members of the immunoglobulin supergene family (such as N-CAM and PECAM); and claudins. Preferred CAR sequences for use within such a modulator include HAV, RGD, and CAR sequences of claudins, VE-cadherin, dsg and dsc.

A pharmaceutical composition may also, or alternatively, contain one or more drugs, which may be linked to a modulating agent or may be free within the composition. Virtually any drug may be administered in combination with a modulating agent as described herein, for a variety of purposes as described below. Examples of types of drugs that may be administered with a modulating agent include analgesics, anesthetics, antianginals, antifungals, antibiotics, anticancer drugs (e.g., taxol or mitomycin C), antiinflammatories (e.g., ibuprofen and indomethacin), anthelmintics, antidepressants, antidotes, antiemetics, antihistamines, antihypertensives, antimalarials, antimicrotubule agents (e.g., colchicine or vinca alkaloids), antimigraine agents, antimicrobials, antiphsychotics, antipyretics, antiseptics, anti-signaling agents (e.g., protein kinase C inhibitors or inhibitors of intracellular calcium mobilization), antiarthritics, antithrombin agents, antituberculotics, antitussives, antivirals, appetite suppressants, cardioactive drugs, chemical dependency drugs, cathartics, chemotherapeutic agents, coronary, cerebral or peripheral vasodilators, contraceptive agents, depressants, diuretics, expectorants, growth factors, hormonal agents, hypnotics, immunosuppression agents, narcotic antagonists, parasympathomimetics, sedatives, stimulants, sympathomimetics, toxins (e.g., cholera toxin), tranquilizers and urinary antiinfectives.

For imaging purposes, any of a variety of diagnostic agents may be incorporated into a pharmaceutical composition, either linked to a modulating agent or free within the composition. Diagnostic agents include any substance administered to illuminate a physiological function within a patient, while leaving other physiological functions generally unaffected. Diagnostic agents include metals, radioactive isotopes and radioopaque agents (e.g., gallium, technetium, indium, strontium, iodine, barium, bromine and phosphorus-containing compounds), radiolucent agents, contrast agents, dyes (e.g., fluorescent dyes and chromophores) and enzymes that catalyze a colorimetric or fluorometric reaction. In general, such agents may be attached using a variety of techniques as described above, and may be present in any orientation.

The compositions described herein may be administered as part of a sustained release formulation (i.e., a formulation such as a capsule or sponge that effects a slow release of modulating agent following administration). Such formulations may generally be prepared using well known technology and administered by, for example, oral, rectal or subcutaneous implantation, or by implantation at the desired target site. Sustained-release formulations may contain a modulating agent dispersed in a carrier matrix and/or contained within a reservoir surrounded by a rate controlling membrane (see, e.g., European Patent Application 710,491 A). Carriers for use within such formulations are biocompatible, and may also be biodegradable; preferably the formulation provides a relatively constant level of modulating agent release. The amount of modulating agent contained within a sustained release formulation depends upon the site of implantation, the rate and expected duration of release and the nature of the condition to be treated or prevented.

Pharmaceutical compositions of the present invention may be administered in a manner appropriate to the disease to be treated (or prevented). Appropriate dosages and a suitable duration and frequency of administration will be determined by such factors as the condition of the patient, the type and severity of the patient's disease and the method of administration. In general, an appropriate dosage and treatment regimen provides the modulating agent(s) in an amount sufficient to provide therapeutic and/or prophylactic benefit. Within particularly preferred embodiments of the invention, a modulating agent or pharmaceutical composition as described herein may be administered at a dosage ranging from 0.001 to 50 mg/kg body weight, preferably from 0.1 to 20 mg/kg, on a regimen of single or multiple daily doses. For topical administration, a cream typically comprises an amount of modulating agent ranging from 0.00001% to 1%, preferably from 0.0001% to 0.2% and more preferably from 0.01% to 0.1%. Fluid compositions typically contain an amount of modulating agent ranging from 10 ng/ml to 5 mg/ml, preferably from 10 μg to 2 mg/mL. Appropriate dosages may generally be determined using experimental models and/or clinical trials. In general, the use of the minimum dosage that is sufficient to provide effective therapy is preferred. Patients may generally be monitored for therapeutic effectiveness using assays suitable for the condition being treated or prevented, which will be familiar to those of ordinary skill in the art.

MODULATING AGENT METHODS OF USE

In general, the modulating agents and compositions described herein may be used for modulating the adhesion of occludin-expressing cells in vitro and/or in vivo. As noted above, modulating agents for purposes that involve the disruption of occludin-mediated cell adhesion may comprise an occludin CAR sequence, multiple occludin CAR sequences in close proximity and/or an antibody (or an antigen-binding fragment thereof) that recognizes the occludin CAR sequence. When it is desirable to also disrupt cell adhesion mediated by other adhesion molecules, a modulating agent may additionally comprise one or more CAR sequences bound by such adhesion molecules (and/or antibodies or fragments thereof that bind such sequences), preferably separated from each other and from the occludin CAR sequence by linkers. As noted above, such linkers may or may not comprise one or more amino acids. For enhancing cell adhesion, a modulating agent may contain multiple occludin CAR sequences or antibodies (or fragments), preferably separated by linkers, and/or may be linked to a single molecule or to a support material as described above.

Certain methods involving the disruption of cell adhesion as described herein have an advantage over prior techniques in that they permit the passage of molecules that are large and/or charged across barriers of occludin-expressing cells. As described in greater detail below, modulating agents as described herein may also be used to disrupt or enhance cell adhesion in a variety of other contexts. Within each of the methods described herein, one or more modulating agents may generally be administered alone, or within a pharmaceutical composition. In each specific method described herein, as noted above, a targeting agent may be employed to increase the local concentration of modulating agent at the target site.

The present invention also provides methods for increasing vasopermeability in a mammal by administering one or more modulating agents or pharmaceutical compositions. It has been found, within the context of the present invention, that endothelial cell adhesion can be disrupted by linear and

cyclic peptides containing the occludin CAR sequence, LYHY (SEQ ID NO:1). Within blood vessels, endothelial cell adhesion results in decreased vascular permeability. Accordingly, modulating agents that disrupt occludin-mediated endothelial cell adhesion as described herein, can increase vascular permeability and thus may facilitate drug delivery to previously inaccessible tissues, such as the brain.

Certain preferred modulating agents for use within such methods are H-QYLYHYCVVD-OH (SEQ ID NO:2) and H-CLYHYC-OH (SEQ ID NO:3) and modulating agents comprising such sequences or derivatives thereof having one or more C-terminal, N-terminal and/or side chain modifications. Preferred antibody modulating agents include Fab fragments directed against either H-QYLYHYCVVD-OH (SEQ ID NO:2) or H-CLYHYC-OH (SEQ ID NO:3). In one particularly preferred embodiment, a modulating agent is capable of disrupting cell adhesion mediated by multiple adhesion molecules. For example, a single branched modulating agent (or multiple agents linked to a single molecule or support material) may disrupt occludin and cadherin mediated cell adhesion, thereby disrupting tight junctions and adherens junctions. Multi-functional modulating agents comprising the occludin CAR sequence LYHY (SEQ ID NO:1) joined (preferable by a linker) to one or more of a classical cadherin CAR sequence, a claudin CAR sequence, an OB-cadherin CAR sequence and/or a VE-cadherin CAR sequence are also preferred. Alternatively, a separate modulator of non-occludin-mediated cell adhesion may be administered in conjunction with the modulating agent(s), either within the same pharmaceutical composition or separately. Preferred antibody modulating agents that may be used in conjunction with the occludin modulating agents include Fab fragments directed against an N-cadherin CAR sequence, such as FHLRAHAVDINGNQV-NH_2 (SEQ ID NO:4).

Within certain embodiments, preferred modulating agents for use within such methods include peptides capable of decreasing both endothelial and tumor cell adhesion. Such modulating agents may be used to facilitate the penetration of anti-tumor therapeutic or diagnostic agents (e.g., monoclonal antibodies) through endothelial cell permeability barriers and tumor barriers. In one particularly preferred embodiment, a modulating agent is capable of disrupting cell adhesion mediated by multiple adhesion molecules. For example, a single branched modulating agent (or multiple agents linked to a single molecule or support material) may disrupt occludin, claudin, VE-cadherin, Dsc and Dsg mediated cell adhesion. Alternatively, a separate modulator of non-occludin-mediated cell adhesion may be administered in conjunction with the modulating agent(s), either within the same pharmaceutical composition or separately. Preferred antibody modulating agents that may be used in conjunction with the occludin modulating agents include Fab fragments directed against either an N-cadherin CAR sequence, such as FHLRAHAVDINGNQV-NH_2 (SEQ ID NO:4), or an E-cadherin CAR sequence, such as LFSHAVSSNG-NH_2 (SEQ ID NO:39).

Treatment with a modulating agent may be appropriate, for example, prior to administration of an anti-tumor therapeutic or diagnostic agent (e.g., a monoclonal antibody or other macromolecule), an antimicrobial agent or an anti-inflammatory agent, in order to increase the concentration of such agents in the vicinity of the target tumor, organism or inflammation without increasing the overall dose to the patient. Modulating agents for use within such methods may be linked to a targeting agent to further increase the local concentration of modulating agent, although systemic administration of a vasoactive agent even in the absence of a targeting agent increases the perfusion of certain tumors relative to other tissues. Suitable targeting agents include antibodies and other molecules that specifically bind to tumor cells or to components of structurally abnormal blood vessels. For example, a targeting agent may be an antibody that binds to a fibrin degradation product or a cell enzyme such as a peroxidase that is released by granulocytes or other cells in necrotic or inflamed tissues.

Administration via intravenous injection or transdermal administration is generally preferred. Effective dosages are generally sufficient to increase localization of a subsequently administered diagnostic or therapeutic agent to an extent that improves the clinical efficacy of therapy of accuracy of diagnosis to a statistically significant degree. Comparison may be made between treated and untreated tumor host animals to whom equivalent doses of the diagnostic or therapeutic agent are administered. In general, dosages range as described above.

Within further aspects, the present invention provides methods in which cell adhesion is diminished. In one such aspect, methods for reducing unwanted cellular adhesion by administering a modulating agent are provided. Unwanted cellular adhesion can occur between tumor cells, between tumor cells and normal cells or between normal cells as a result of surgery, injury, chemotherapy, disease, inflammation or other condition jeopardizing cell viability or function. Preferred modulating agents for use within such methods include H-QYLYHYCVVD-OH (SEQ ID NO:2) and H-CLYHYC-OH (SEQ ID NO:3) and modulating agents comprising such sequences or derivatives thereof. Preferred antibody modulating agents include Fab fragments directed against either H-QYLYHYCVVD-OH (SEQ ID NO:2) or H-CLYHYC-OH (SEQ ID NO:3). In addition, a modulating agent may comprise one or more of a claudin CAR sequence, a CAR sequence for a nonclassical cadherin (such as VE-cadherin, OB-cadherin, dsc or dsg), RGD sequence, and/or HAV sequence separated from an occludin CAR sequence via a linker. Alternatively, separate modulators of cadherin- and integrin-mediated cell adhesion may be administered in conjunction with the modulating agent(s), either within the same pharmaceutical composition or separately. Topical administration of the modulating agent(s) is generally preferred, but other means may also be employed. Preferably, a fluid composition for topical administration (comprising, for example, physiological saline) comprises an amount of modulating agent as described above, and more preferably from 10 μg/mL to 1 mg/mL. Creams may generally be formulated as described above. Topical administration in the surgical field may be given once at the end of surgery by irrigation of the wound or as an intermittent or continuous irrigation with the use of surgical drains in the post-operative period or by the use of drains specifically inserted in an area of inflammation, injury or disease in cases where surgery does not need to be performed. Alternatively, parenteral or transcutaneous administration may be used to achieve similar results.

Within another such aspect, methods are provided for enhancing the delivery of a drug through the skin of a mammal. Transdermal delivery of drugs is a convenient and non-invasive method that can be used to maintain relatively constant blood levels of a drug. In general, to facilitate drug delivery via the skin, it is necessary to perturb adhesion between the epithelial cells (keratinocytes) and the endothelial cells of the microvasculature. Using currently available techniques, only small, uncharged molecules may be delivered across skin in vivo. The methods described herein

are not subject to the same degree of limitation. Accordingly, a wide variety of drugs may be transported across the epithelial and endothelial cell layers of skin, for systemic or topical administration. Such drugs may be delivered to melanomas or may enter the blood stream of the mammal for delivery to other sites within the body.

To enhance the delivery of a drug through the skin, a modulating agent as described herein and a drug are contacted with the skin surface. Preferred modulating agents for use within such methods include H-QYLYHYCVVD-OH (SEQ ID NO:2) and H-CLYHYC-OH (SEQ ID NO:3) and modulating agents comprising such sequences or derivatives thereof. Preferred antibody modulating agents include Fab fragments directed against either H-QYLYHYCVVD-OH (SEQ ID NO:2) or H-CLYHYC-OH (SEQ ID NO:3). Multifunctional modulating agents comprising an occludin CAR sequence linked to one or more of a claudin CAR sequence, VE-cadherin CAR sequence, dsc CAR sequence, dsg CAR sequence, RGD sequence, and/or HAV sequence may also be used to disrupt cell adhesion. Alternatively, a separate modulator of non-occludin-mediated cell adhesion may be administered in conjunction with the modulating agent(s), either within the same pharmaceutical composition or separately. Contact may be achieved by direct application of the modulating agent, generally within a composition formulated as a cream or gel, or using any of a variety of skin contact devices for transdermal application (such as those described in European Patent Application No. 566,816 A; U.S. Pat. No. 5,613,958; U.S. Pat. No. 5,505,956). A skin patch provides a convenient method of administration (particularly for slow-release formulations). Such patches may contain a reservoir of modulating agent and drug separated from the skin by a membrane through which the drug diffuses. Within other patch designs, the modulating agent and drug may be dissolved or suspended in a polymer or adhesive matrix that is then placed in direct contact with the patient's skin. The modulating agent and drug may then diffuse from the matrix into the skin. Modulating agent(s) and drug(s) may be contained within the same composition or skin patch, or may be separately administered, although administration at the same time and site is preferred. In general, the amount of modulating agent administered via the skin varies with the nature of the condition to be treated or prevented, but may vary as described above. Such levels may be achieved by appropriate adjustments to the device used, or by applying a cream formulated as described above. Transfer of the drug across the skin and to the target tissue may be predicted based on in vitro studies using, for example, a Franz cell apparatus, and evaluated in vivo by appropriate means that will be apparent to those of ordinary skill in the art. As an example, monitoring of the serum level of the administered drug over time provides an easy measure of the drug transfer across the skin.

Transdermal drug delivery as described herein is particularly useful in situations in which a constant rate of drug delivery is desired, to avoid fluctuating blood levels of a drug. For example, morphine is an analgesic commonly used immediately following surgery. When given intermittently in a parenteral form (intramuscular, intravenous), the patient usually feels sleepy during the first hour, is well during the next 2 hours and is in pain during the last hour because the blood level goes up quickly after the injection and goes down below the desirable level before the 4 hour interval prescribed for re-injection is reached. Transdermal administration as described herein permits the maintenance of constant levels for long periods of time (e.g., days), which allows adequate pain control and mental alertness at the same time. Insulin provides another such example. Many diabetic patients need to maintain a constant baseline level of insulin which is different from their needs at the time of meals. The baseline level may be maintained using transdermal administration of insulin, as described herein. Antibiotics may also be administered at a constant rate, maintaining adequate bactericidal blood levels, while avoiding the high levels that are often responsible for the toxicity (e.g., levels of gentamycin that are too high typically result in renal toxicity).

Drug delivery by the methods of the present invention also provide a more convenient method of drug administration. For example, it is often particularly difficult to administer parenteral drugs to newborns and infants because of the difficulty associated with finding veins of acceptable caliber to catheterize. However, newborns and infants often have a relatively large skin surface as compared to adults. Transdermal drug delivery permits easier management of such patients and allows certain types of care that can presently be given only in hospitals to be given at home. Other patients who typically have similar difficulties with venous catheterization are patients undergoing chemotherapy or patients on dialysis. In addition, for patients undergoing prolonged therapy, transdermal administration as described herein is more convenient than parenteral administration.

Transdermal administration as described herein also allows the gastrointestinal tract to be bypassed in situations where parenteral uses would not be practical. For example, there is a growing need for methods suitable for administration of therapeutic small peptides and proteins, which are typically digested within the gastrointestinal tract. The methods described herein permit administration of such compounds and allow easy administration over long periods of time. Patients who have problems with absorption through their gastrointestinal tract because of prolonged ileus or specific gastrointestinal diseases limiting drug absorption may also benefit from drugs formulated for transdermal application as described herein.

Further, there are many clinical situations where it is difficult to maintain compliance. For example, patients with mental problems (e.g., patients with Alzheimer's disease or psychosis) are easier to manage if a constant delivery rate of drug is provided without having to rely on their ability to take their medication at specific times of the day. Also patients who simply forget to take their drugs as prescribed are less likely to do so if they merely have to put on a skin patch periodically (e.g., every 3 days). Patients with diseases that are without symptoms, like patients with hypertension, are especially at risk of forgetting to take their medication as prescribed.

For patients taking multiple drugs, devices for transdermal application such as skin patches may be formulated with combinations of drugs that are frequently used together. For example, many heart failure patients are given digoxin in combination with furosemide. The combination of both drugs into a single skin patch facilitates administration, reduces the risk of errors (taking the correct pills at the appropriate time is often confusing to older people), reduces the psychological strain of taking "so many pills," reduces skipped dosage because of irregular activities and improves compliance.

The methods described herein are particularly applicable to humans, but also have a variety of veterinary uses, such as the administration of growth factors or hormones (e.g., for fertility control) to an animal.

As noted above, a wide variety of drugs may be administered according to the methods provided herein. Some

examples of drug categories that may be administered transdermally include anti-inflammatory drugs (e.g., in arthritis and in other condition) such as all NSAID, indomethacin, prednisone, etc.; analgesics (especially when oral absorption is not possible, such as after surgery, and when parenteral administration is not convenient or desirable), including morphine, codeine, Demerol, acetaminophen and combinations of these (e.g., codeine plus acetaminophen); antibiotics such as Vancomycin (which is not absorbed by the GI tract and is frequently given intravenously) or a combination of INH and Rifampicin (e.g., for tuberculosis); anticoagulants such as heparin (which is not well absorbed by the GI tract and is generally given parenterally, resulting in fluctuation in the blood levels with an increased risk of bleeding at high levels and risks of inefficacy at lower levels) and Warfarin (which is absorbed by the GI tract but cannot be administered immediately after abdominal surgery because of the normal ileus following the procedure); antidepressants (e.g., in situations where compliance is an issue as in Alzheimer's disease or when maintaining stable blood levels results in a significant reduction of anti-cholinergic side effects and better tolerance by patients), such as amitriptylin, imipramin, prozac, etc.; antihypertensive drugs (e.g., to improve compliance and reduce side effects associated with fluctuating blood levels), such as diuretics and beta-blockers (which can be administered by the same patch; e.g., furosemide and propanolol); antipsychotics (e.g., to facilitate compliance and make it easier for care giver and family members to make sure that the drug is received), such as haloperidol and chlorpromazine; and anxiolytics or sedatives (e.g., to avoid the reduction of alertness related to high blood levels after oral administration and allow a continual benefit throughout the day by maintaining therapeutic levels constant).

Numerous other drugs may be administered as described herein, including naturally occurring and synthetic hormones, growth factors, proteins and peptides. For example, insulin and human growth hormone, growth factors like erythropoietin, interleukins and inteferons may be delivered via the skin.

Kits for administering a drug via the skin of a mammal are also provided within the present invention. Such kits generally comprise a device for transdermal application (e.g., a skin patch) in combination with, or impregnated with, one or more modulating agents. A drug may additionally be included within such kits.

Within a related aspect, the use of modulating agents as described herein to increase skin permeability may also facilitate sampling of the blood compartment by passive diffusion, permitting detection and/or measurement of the levels of specific molecules circulating in the blood. For example, application of one or more modulating agents to the skin, via a skin patch as described herein, permits the patch to function like a sponge to accumulate a small quantity of fluid containing a representative sample of the serum. The patch is then removed after a specified amount of time and analyzed by suitable techniques for the compound of interest (e.g., a medication, hormone, growth factor, metabolite or marker). Alternatively, a patch may be impregnated with reagents to permit a color change if a specific substance (e.g. an enzyme) is detected. Substances that can be detected in this manner include, but are not limited to, illegal drugs such as cocaine, HIV enzymes, glucose and PSA. This technology is of particular benefit for home testing kits.

Within a further aspect, methods are provided for enhancing delivery of a drug to a tumor in a mammal, comprising administering a modulating agent in combination with a drug to a tumor-bearing mammal. Preferred modulating agents for use within such methods include H-QYLYHYCVVD-OH (SEQ ID NO:2) and H-CLYHYC-OH (SEQ ID NO:3) and modulating agents comprising such sequences or derivatives thereof. Preferred antibody modulating agents include Fab fragments directed against either H-QYLYHYCVVD-OH (SEQ ID NO:2) or H-CLYHYC-OH (SEQ ID NO:3). In one particularly preferred embodiment, a modulating agent is capable of disrupting cell adhesion mediated by multiple adhesion molecules. For example, a single branched modulating agent (or multiple agents linked to a single molecule or support material) may disrupt occludin, classical cadherin, integrin, and nonclassical cadherin (e.g., Dsc and Dsg) mediated cell adhesion, thereby disrupting tight junctions, adherens junctions, and desmosomes. Multifunctional modulating agents comprising an occludin CAR sequence linked to an RGD sequence and/or CAR sequence(s) for one or more of a classical cadherin, claudin or nonclassical cadherin (e.g., OB-cadherin, VE-cadherin, dsc or dsg) may be used to disrupt cell adhesion. Alternatively, a separate modulator of non-occludin-mediated cell adhesion may be administered in conjunction with the modulating agent(s), either within the same pharmaceutical composition or separately. Preferred antibody modulating agents that may be used in conjunction with the occludin modulating agents include Fab fragments directed against either an N-cadherin CAR sequence (such as FHLRAHAVDINGNQV-NH_2; SEQ ID NO:4) or an E-cadherin CAR sequence LFSHAVSSNG-NH_2 (SEQ ID NO:39).

Preferably, the modulating agent and the drug are formulated within the same composition or drug delivery device prior to administration. In general, a modulating agent may enhance drug delivery to any tumor, and the method of administration may be chosen based on the type of target tumor. For example, injection or topical administration as described above may be preferred for melanomas and other accessible tumors (e.g., metastases from primary ovarian tumors may be treated by flushing the peritoneal cavity with the composition). Other tumors (e.g., bladder tumors) may be treated by injection of the modulating agent and the drug (such as mitomycin C) into the site of the tumor. In other instances, the composition may be administered systemically, and targeted to the tumor using any of a variety of specific targeting agents. Suitable drugs may be identified by those of ordinary skill in the art based upon the type of cancer to be treated (e.g., mitomycin C for bladder cancer). In general, the amount of modulating agent administered varies with the method of administration and the nature of the tumor, within the typical ranges provided above, preferably ranging from about 1 μg/mL to about 2 mg/mL, and more preferably from about 10 μg/mL to 1 mg/mL. Transfer of the drug to the target tumor may be evaluated by appropriate means that will be apparent to those of ordinary skill in the art. Drugs may also be labeled (e.g., using radionuclides) to permit direct observation of transfer to the target tumor using standard imaging techniques.

Within a related aspect, the present invention provides methods for treating cancer and/or inhibiting metastasis in a mammal. Cancer tumors are solid masses of cells, growing out of control, which require nourishment via blood vessels. The formation of new capillaries is a prerequisite for tumor growth and the emergence of metastases. Administration of modulating agents as described herein may disrupt the growth of such blood vessels, thereby providing effective therapy for the cancer and/or inhibiting metastasis. Modu-

lating agents may also be used to treat leukemias. Preferred modulating agents for use within such methods include H-QYLYHYCVVD-OH (SEQ ID NO:2) and H-CLYHYC-OH (SEQ ID NO:3) and modulating agents comprising such sequences or derivatives thereof. Preferred antibody modulating agents include Fab fragments directed against either H-QYLYHYCVVD-OH (SEQ ID NO:2) or H-CLYHYC-OH (SEQ ID NO:3). In one particularly preferred embodiment, a modulating agent is capable of disrupting cell adhesion mediated by multiple adhesion molecules. For example, a single branched modulating agent (or multiple agents linked to a single molecule or support material) may disrupt occludin, classical cadherin, integrin, Dsc and Dsg mediated cell adhesion, thereby disrupting tight junctions, adherens junctions, focal contacts and desmosomes. Multifunctional modulating agents comprising the occludin CAR sequence linked to one or more of a claudin CAR sequence, VE-cadherin CAR sequence, dsc CAR sequence, dsg CAR sequence, RGD sequence, OB-cadherin CAR sequence and/or HAV sequence may be used to disrupt cell adhesion. Alternatively, a separate modulator of non-occludin-mediated cell adhesion may be administered in conjunction with the modulating agent(s), either within the same pharmaceutical composition or separately. Preferred antibody modulating agents that may be used in conjunction with the occludin modulating agents include Fab fragments directed against either an N-cadherin CAR sequence, such as FHLRAHAVDINGNQV-NH_2 (SEQ ID NO:4), or an E-cadherin CAR sequence, such as LFSHAVSSNG-NH_2 (SEQ ID NO:39). A modulating agent may be administered alone (e.g., via the skin) or within a pharmaceutical composition. For melanomas and certain other accessible tumors, injection or topical administration as described above may be preferred. For ovarian cancers, flushing the peritoneal cavity with a composition comprising one or more modulating agents may prevent metastasis of ovarian tumor cells. Other tumors (e.g., bladder tumors, bronchial tumors or tracheal tumors) may be treated by injection of the modulating agent into the cavity. In other instances, the composition may be administered systemically, and targeted to the tumor using any of a variety of specific targeting agents, as described above. In general, the amount of modulating agent administered varies depending upon the method of administration and the nature of the cancer, but may vary within the ranges identified above. The effectiveness of the cancer treatment or inhibition of metastasis may be evaluated using well known clinical observations, such as monitoring the level of serum tumor markers (e.g., CEA or PSA).

Within a further related aspect, a modulating agent may be used to inhibit angiogenesis (i.e., the growth of blood vessels from pre-existing blood vessels) in a mammal. Inhibition of angiogenesis may be beneficial, for example, in patients afflicted with diseases such as cancer or arthritis. Preferred modulating agents for use within such methods include H-QYLYHYCVVD-OH (SEQ ID NO:2) and H-CLYHYC-OH (SEQ ID NO:3) and modulating agents comprising such sequences or derivatives thereof. Preferred antibody modulating agents include Fab fragments directed against either H-QYLYHYCVVD-OH (SEQ ID NO:2) or H-CLYHYC-OH (SEQ ID NO:3). In one particularly preferred embodiment, a modulating agent is capable of disrupting cell adhesion mediated by multiple adhesion molecules. For example, a single branched modulating agent (or multiple agents linked to a single molecule or support material) may disrupt occludin, classical cadherin, and integrin mediated cell adhesion, thereby disrupting tight junctions, adherens junctions, and focal contacts. Multifunctional modulating agents comprising the occludin CAR sequence linked to one or more of a claudin CAR sequence, VE-cadherin CAR sequence, dsc CAR sequence, dsg CAR sequence, RGD sequence, and/or HAV sequence may be used to disrupt cell adhesion. Alternatively, a separate modulator of non-occludin-mediated cell adhesion may be administered in conjunction with the modulating agent(s), either within the same pharmaceutical composition or separately. Preferred antibody modulating agents that may be used in conjunction with the occludin modulating agents include Fab fragments directed against an N-cadherin CAR sequence, such as FHLRAHAVDINGNQV-NH_2 (SEQ ID NO:4).

The effect of a particular modulating agent on angiogenesis may generally be determined by evaluating the effect of the agent on blood vessel formation. Such a determination may generally be performed, for example, using a chick chorioallantoic membrane assay (Iruela-Arispe et al., *Molecular Biology of the Cell* 6:327–343, 1995). Briefly, a modulating agent may be embedded in a mesh composed of vitrogen at one or more concentrations (e.g., ranging from about 5 to 50 μg/mesh). The mesh(es) may then be applied to chick chorioallantoic membranes. After 24 hours, the effect of the modulating agent may be determined using computer assisted morphometric analysis. A modulating agent should inhibit angiogenesis by at least 25% at a concentration of 50 μg/mesh.

The addition of a targeting agent as described above may be beneficial, particularly when the administration is systemic. Suitable modes of administration and dosages depend upon the condition to be prevented or treated but, in general, administration by injection is appropriate. Dosages may vary as described above. The effectiveness of the inhibition may be evaluated grossly by assessing the inability of the tumors to maintain their growth and microscopically by observing an absence of nerves at the periphery of the tumor.

In yet another related aspect, the present invention provides methods for inducing apoptosis in an occludin-expressing cell. In general, patients afflicted with cancer may benefit from such treatment. Preferred modulating agents for use within such methods include H-QYLYHYCVVD-OH (SEQ ID NO:2) and H-CLYHYC-OH (SEQ ID NO:3) and modulating agents comprising such sequences or derivatives thereof. Preferred antibody modulating agents include Fab fragments directed against either H-QYLYHYCVVD-OH (SEQ ID NO:2) or H-CLYIIYC-OH (SEQ ID NO:3). In one particularly preferred embodiment, a modulating agent is capable of disrupting cell adhesion mediated by multiple adhesion molecules. For example, a single branched modulating agent (or multiple agents linked to a single molecule or support material) may disrupt occludin, classical cadherin, and integrin mediated cell adhesion, thereby disrupting tight junctions, adherens junctions, and focal contacts. Multifunctional modulating agents comprising the occludin CAR sequence linked to one or more of a claudin CAR sequence, nonclassical cadherin CAR sequence (e.g., VE-cadherin, OB-cadherin, dsc or dsg), RGD sequence, and/or HAV sequence may be used to disrupt cell adhesion. Alternatively, a separate modulator of non-occludin-mediated cell adhesion may be administered in conjunction with the modulating agent(s), either within the same pharmaceutical composition or separately. Preferred antibody modulating agents that may be used in conjunction with the occludin modulating agents include Fab fragments directed against either an N-cadherin CAR sequence, such as

FHLRAHAVDINGNQV-NH_2 (SEQ ID NO:4), or an E-cadherin CAR sequence, such as LFSHAVSSNG-NH_2 (SEQ ID NO:39).

Administration of modulating agents to induce apoptosis may be topical, via injection or by other means, and the addition of a targeting agent may be beneficial, particularly when the administration is systemic. Suitable modes of administration and dosages depend upon the location and nature of the cells for which induction of apoptosis is desired but, in general, dosages may vary as described above. A biopsy may be performed to evaluate the level of induction of apoptosis.

The present invention also provides methods for enhancing drug delivery to the central nervous system of a mammal. The blood/brain barrier is largely impermeable to most neuroactive agents, and delivery of drugs to the brain of a mammal often requires invasive procedures. Using a modulating agent as described herein, however, delivery may be by, for example, systemic administration of a modulating agent-drug-targeting agent combination, injection of a modulating agent (alone or in combination with a drug and/or targeting agent) into the carotid artery or application of a skin patch comprising a modulating agent to the head of the patient. Certain preferred modulating agents for use within such methods are H-QYLYHYCVVD-OH (SEQ ID NO:2) and H-CLYHYC-OH (SEQ ID NO:3) and modulating agents comprising such sequences or derivatives thereof. Preferred antibody modulating agents include Fab fragments directed against either H-QYLYHYCVVD-OH (SEQ ID NO:2) or H-CLYHYC-OH (SEQ ID NO:3). In one particularly preferred embodiment, a modulating agent is capable of disrupting cell adhesion mediated by multiple adhesion molecules. For example, a single branched modulating agent (or multiple agents linked to a single molecule or support material) may disrupt occludin and cadherin mediated cell adhesion, thereby disrupting tight junctions and adherens junctions. Multifunctional modulating agents comprising the occludin CAR sequence linked to one or more of a claudin CAR sequence, VE-cadherin CAR sequence, OB-cadherin CAR sequence and/or HAV sequence may be used to disrupt cell adhesion. Alternatively, a separate modulator of non-occludin-mediated cell adhesion may be administered in conjunction with the modulating agent(s), either within the same pharmaceutical composition or separately. Preferred antibody modulating agents that may be used in conjunction with the occludin modulating agents include Fab fragments directed against the N-cadherin CAR sequence FHLRAHAVDINGNQV-NH_2 (SEQ ID NO:4).

In general, the amount of modulating agent administered varies as described above, and with the method of administration and the nature of the condition to be treated or prevented. Transfer of the drug to the central nervous system may be evaluated by appropriate means that will be apparent to those of ordinary skill in the art, such as magnetic resonance imaging (MRI) or PET scan (positron emitted tomography).

Within further aspects, modulating agents as described herein may be used for modulating the immune system of a mammal in any of several ways. Modulating agents may generally be used to modulate specific steps within cellular interactions during an immune response or during the dissemination of malignant lymphocytes. For example, a modulating agent as described herein may be used to treat diseases associated with excessive generation of otherwise normal T cells. Without wishing to be bound by any particular theory, it is believed that the interaction of occludin on maturing T cells and B cell subsets contributes to protection of these cells from programmed cell death. A modulating agent may decrease such interactions, leading to the induction of programmed cell death. Accordingly, modulating agents may be used to treat certain types of diabetes and rheumatoid arthritis, particularly in young children where the cadherin expression on thymic pre-T cells is greatest.

Modulating agents may also be administered to patients afflicted with certain skin disorders (such as cutaneous lymphomas), acute B cell leukemia and excessive immune reactions involving the humoral immune system and generation of immunoglobulins, such as allergic responses and antibody-mediated graft rejection. In addition, patients with circulating cadherin-positive malignant cells (e.g., during regimes where chemotherapy or radiation therapy is eliminating a major portion of the malignant cells in bone marrow and other lymphoid tissue) may benefit from treatment with a modulating agent. Such treatment may also benefit patients undergoing transplantation with peripheral blood stem cells.

Certain preferred modulating agents for use within such methods include those that comprise one or more additional CAR sequences, such as a claudin CAR sequence, HAV, RGD, a VE-cadherin CAR sequence and/or KYSFNYDGSE (SEQ ID NO:11). As noted above, such additional sequence(s) may be separated from an occludin CAR sequence via a linker. Alternatively, a separate modulator of cadherin-, claudin-, integrin- and/or N-CAM-mediated cell adhesion may be administered in conjunction with the modulating agent(s), either within the same pharmaceutical composition or separately.

Within the above methods, the modulating agent(s) are preferably administered systemically (usually by injection) or topically. A modulating agent may be linked to a targeting agent. For example, targeting to the bone marrow may be beneficial. A suitable dosage is sufficient to effect a statistically significant reduction in the population of B and/or T cells that express cadherin and/or an improvement in the clinical manifestation of the disease being treated. Typical dosages generally range as described above.

In certain other aspects, the present invention provides methods for enhancing adhesion of occludin-expressing cells. Within certain embodiments, a modulating agent may be linked to a solid support, resulting in a matrix that comprises multiple modulating agents. Within one such embodiment, the support is a polymeric matrix to which modulating agents and molecules comprising other CAR sequence(s) are attached (e.g., modulating agents and molecules comprising HAV and RGD sequences may be attached to the same matrix, preferably in an alternating pattern). Such matrices may be used in contexts in which it is desirable to enhance adhesion mediated by multiple cell adhesion molecules. Alternatively, the modulating agent itself may comprise multiple occludin CAR sequences or antibodies (or fragments thereof), separated by linkers as described above. Either way, the modulating agent(s) function as a "biological glue" to bind multiple occludin-expressing cells within a variety of contexts.

Within one such aspect, modulating agents comprising multiple occludin CAR sequences and/or multiple modulating agents linked to a single molecule or support material may be used to enhance wound healing and/or reduce scar tissue in a mammal. Peptides that may be linked to a support, and/or to one another via a linker, to generate a suitable modulating agent include, but are not limited to, H-QYLYHYCVVD-OH (SEQ ID NO:2) and H-CLYHYC-OH (SEQ ID NO:3) and modulating agents com-

prising such sequences or derivatives thereof. Preferred antibody modulating agents include Fab fragments directed against either H-QYLYHYCVVD-OH (SEQ ID NO:2) or H-CLYHYC-OH (SEQ ID NO:3). Modulating agents that are linked to a biocompatible and biodegradable matrix such as cellulose or collagen are particularly preferred. For use within such methods, a modulating agent should have a free amino or hydroxyl group. The modulating agents are generally administered topically to the wound, where they may facilitate closure of the wound and may augment, or even replace, stitches. Similarly, administration of matrix-linked modulating agents may facilitate cell adhesion in skin grafting and prosthetic implants, and may prolong the duration and usefulness of collagen injection. In general, the amount of matrix-linked modulating agent administered to a wound, graft or implant site varies with the severity of the wound and/or the nature of the wound, graft, or implant, but may vary as discussed above. Multifunctional modulating agents comprising the occludin CAR sequence linked to one or more of a claudin CAR sequence, nonclassical cadherin CAR sequence (e.g., VE-cadherin, OB-cadherin, dsc or dsg), RGD sequence, and/or HAV sequence may be used as potent stimulators of wound healing and/or to reduce scar tissue. Alternatively, one or more separate modulators of cadherin-, claudin-, integrin-, Dsc- and/or Dsg-mediated cell adhesion may be administered in conjunction with the modulating agent(s), either within the same pharmaceutical composition or separately.

Within another aspect, one or more modulating agents may be linked to the interior surface of a tissue culture plate or other cell culture support, such as for use in a bioreactor. Such linkage may be performed by any suitable technique, as described above. Modulating agents linked in this fashion may generally be used to immobilize occludin-expressing cells. For example, dishes or plates coated with one or more modulating agents may be used to immobilize occludin-expressing cells within a variety of assays and screens. Within bioreactors (i.e., systems for large scale production of cells or organoids), modulating agents may generally be used to improve cell attachment and stabilize cell growth. Modulating agents may also be used within bioreactors to support the formation and function of highly differentiated organoids derived, for example, from dispersed populations of fetal mammalian cells. Bioreactors containing biomatrices of modulating agent(s) may also be used to facilitate the production of specific proteins.

Modulating agents as described herein may be used within a variety of bioreactor configurations. In general, a bioreactor is designed with an interior surface area sufficient to support large numbers of adherent cells. This surface area can be provided using membranes, tubes, microtiter wells, columns, hollow fibers, roller bottles, plates, dishes, beads or a combination thereof. A bioreactor may be compartmentalized. The support material within a bioreactor may be any suitable material known in the art; preferably, the support material does not dissolve or swell in water. Preferred support materials include, but are not limited to, synthetic polymers such as acrylics, vinyls, polyethylene, polypropylene, polytetrafluoroethylene, nylons, polyurethanes, polyamides, polysulfones and poly(ethylene terephthalate); ceramics; glass and silica.

Within certain aspects, modulating agents may be used to stimulate the formation of epithelial cell tight junctions. It has been found, within the context of the present invention, that certain peptide modulating agents that inhibit adhesion of endothelial cells, may stimulate adhesion of epithelial cells. Such agents include H-QYLYHYCVVD-COOH (SEQ ID NO:2) and N-Ac-CLYHYC-NH_2 (SEQ ID NO:3), and other such agents may be readily identified using the assays provided herein. Agents that stimulate adhesion of epithelial cells may be used in any context in which such selective stimulation is desirable. For example, diarrhea is known to be the result of toxins that break down tight junctions in intestinal epithelial cells (see, e.g., Philpott et al., *Infect. Immun.* 66:1680–1687, 1998; Spitz et al., *Am. J. Physiol.* 268:G374–379, 1995; Fasano et al., *Proc. Natl. Acad. Sci. USA,* 88:5242–5246, 1991). Modulating agents that stimulate tight junction formation may be administered to patients as described herein to inhibit diarrhea. Such agents may, for example, be administered orally.

Other aspects of the present invention provide methods that employ antibodies raised against the modulating agents for diagnostic and assay purposes. Assays typically involve using an antibody to detect the presence or absence of occludin (free or on the surface of a cell), or proteolytic fragment containing the EC2 domain in a suitable biological sample, such as tumor or normal tissue biopsies, blood, lymph node, serum or urine samples, or other tissue, homogenate, or extract thereof obtained from a patient.

There are a variety of assay formats known to those of ordinary skill in the art for using an antibody to detect a target molecule in a sample. See, e.g., Harlow and Lane, *Antibodies: A Laboratory Manual,* Cold Spring Harbor Laboratory, 1988. For example, the assay may be performed in a Western blot format, wherein a protein preparation from the biological sample is submitted to gel electrophoresis, transferred to a suitable membrane and allowed to react with the antibody. The presence of the antibody on the membrane may then be detected using a suitable detection reagent, as described below.

In another embodiment, the assay involves the use of antibody immobilized on a solid support to bind to the target occludin, or a proteolytic fragment containing the EC2 domain and encompassing the CAR sequence, and remove it from the remainder of the sample. The bound occludin may then be detected using a second antibody or reagent that contains a reporter group. Alternatively, a competitive assay may be utilized, in which the occludin is labeled with a reporter group and allowed to bind to the immobilized antibody after incubation of the antibody with the sample. The extent to which components of the sample inhibit the binding of the labeled occludin to the antibody is indicative of the reactivity of the sample with the immobilized antibody, and as a result, indicative of the level of the occludin in the sample.

The solid support may be any material known to those of ordinary skill in the art to which the antibody may be attached, such as a test well in a microtiter plate, a nitrocellulose filter or another suitable membrane. Alternatively, the support may be a bead or disc, such as glass, fiberglass, latex or a plastic such as polystyrene or polyvinylchloride. The antibody may be immobilized on the solid support using a variety of techniques known to those in the art, which are amply described in the patent and scientific literature.

In certain embodiments, the assay for detection of occludin in a sample is a two-antibody sandwich assay. This assay may be performed by first contacting an antibody that has been immobilized on a solid support, commonly the well of a microtiter plate, with the biological sample, such that the occludin within the sample is allowed to bind to the immobilized antibody (a 30 minute incubation time at room temperature is generally sufficient). Unbound sample is then removed from the immobilized occludin-antibody com-

plexes and a second antibody (containing a reporter group such as an enzyme, dye, radionuclide, luminescent group, fluorescent group or biotin) capable of binding to a different site on the occludin is added. The amount of second antibody that remains bound to the solid support is then determined using a method appropriate for the specific reporter group. The method employed for detecting the reporter group depends upon the nature of the reporter group. For radioactive groups, scintillation counting or autoradiographic methods are generally appropriate. Spectroscopic methods may be used to detect dyes, luminescent groups and fluorescent groups. Biotin may be detected using avidin, coupled to a different reporter group (commonly a radioactive or fluorescent group or an enzyme). Enzyme reporter groups may generally be detected by the addition of substrate (generally for a specific period of time), followed by spectroscopic or other analysis of the reaction products. Standards and standard additions may be used to determine the level of occludin in a sample, using well known techniques.

The present invention also provides kits for use in such immunoassays. Such kits generally comprise one or more antibodies, as described above. In addition, one or more additional compartments or containers of a kit generally enclose elements, such as reagents, buffers and/or wash solutions, to be used in the immunoassay.

Within further aspects, modulating agents or antibodies (or fragments thereof) may be used to facilitate cell identification and sorting in vitro or imaging in vivo, permitting the selection of cells expressing occludin (or different occludin levels). Preferably, the modulating agent(s) or antibodies for use in such methods are linked to a detectable marker. Suitable markers are well known in the art and include radionuclides, luminescent groups, fluorescent groups, enzymes, dyes, constant immunoglobulin domains and biotin. Within one preferred embodiment, a modulating agent linked to a fluorescent marker, such as fluorescein, is contacted with the cells, which are then analyzed by fluorescence activated cell sorting (FACS).

Antibodies or fragments thereof may also be used within screens of combinatorial or other nonpeptide-based libraries to identify other compounds capable of modulating occludin-mediated cell adhesion. Such screens may generally be performed using an ELISA or other method well known to those of ordinary skill in the art that detect compounds with a shape and structure similar to that of the modulating agent. In general, such screens may involve contacting an expression library producing test compounds with an antibody, and detecting the level of antibody bound to the candidate compounds. Compounds for which the antibody has a higher affinity may be further characterized as described herein, to evaluate the ability to modulate occludin-mediated cell adhesion.

The following Examples are offered by way of illustration and not by way of limitation.

EXAMPLE 1

Preparation of Representative Cyclic Peptides

This Example illustrates the solid phase synthesis of representative linear and cyclic peptides as modulating agents.

The peptides were assembled on methylbenzhydrylamine resin (MBHA resin) for the C-terminal amide peptides. The traditional Merrifield resins were used for any C-terminal acid peptides. Bags of a polypropylene mesh material were filled with the resin and soaked in dichloromethane. The resin packets were washed three times with 5% diisopropylethylamine in dichloromethane and then washed with dichloromethane. The packets are then sorted and placed into a Nalgene bottle containing a solution of the amino acid of interest in dichloromethane. An equal amount of diisopropylcarbodiimide (DIC) in dichloromethane was added to activate the coupling reaction. The bottle was shaken for one hour to ensure completion of the reaction. The reaction mixture was discarded and the packets washed with DMF. The N-α-Boc was removed by acidolysis using a 55% TFA in dichloromethane for 30 minutes leaving the TFA salt of the α-amino group. The bags were washed and the synthesis completed by repeating the same procedure while substituting for the corresponding amino acid at the coupling step. Acetylation of the N-terminal was performed by reacting the peptide resins with a solution of acetic anhydride in dichloromethane in the presence of diisopropylethylamine. The peptide was then side-chain deprotected and cleaved from the resin at 0° C. with liquid HF in the presence of anisole as a carbocation scavenger.

The crude peptides were purified by reversed-phase high-performance liquid chromatography. Purified linear precursors of the cyclic peptides were solubilized in 75% acetic acid at a concentration of 2–10 mg/mL. A 10% solution of iodine in methanol was added dropwise until a persistent coloration was obtained. A 5% ascorbic acid solution in water was then added to the mixture until discoloration. The disulfide bridge containing compounds were then purified by HPLC and characterized by analytical HPLC and by mass spectral analysis.

EXAMPLE 2

Establishment of a Model System for Assessing Endothelial Cell Adhesion

This Example illustrates an endothelial cell adhesion assay for evaluating the effects of occludin-modulating agents on endothelial cell adhesion.

A. Cell Culture

Human aortic endothelial cells (HAEC) were cultured on fibronectin (Sigma, St. Louis, Mo.) according to the procedures of Jaffe et al., *J. Clin. Invest.* 52:2745–2756, 1973. Cells were maintained in EGM (endothelial cell growth medium; Clonetics, San Diego, Calif.) and used for experiments at passage 4.

B. Occludin and VE-cadherin Immunolocalization Methods

HAEC were cultured on fibronectin-coated coverslips. Confluent cultures of HAEC were exposed to linear peptides (final concentration 100 μg/ml EGM), or EGM alone for 1 hour. The cells were then fixed for 30 minutes at 4° C. in 95% ethanol, followed by fixation in acetone for 1 minute at 4° C. (Furuse et al., *J. Cell Biol.* 123:1777–1788, 1993). After fixation, the cells were allowed to air dry at room temperature. The cells were probed with either mouse anti-VE-cadherin antibodies (Hemeris, Sassenage, France; diluted 1:250 in 0.1% dried skim milk powder dissolved in PBS), or rabbit anti-occludin antibodies (Zymed, South San Francisco, Calif.; diluted 1:300 in 0.1% dried skim milk powder dissolved in PBS) for 1 hour at 37° C. The cells were then washed with 0.1% dried skim milk powder dissolved in PBS (three washes, 5 minutes/wash), and probed with secondary antibodies (donkey anti-mouse Cy3, or donkey anti-rabbit Cy5 diluted 1:250 in 0.1% dried skim milk powder dissolved in PBS; Jackson Immunoresearch Laboratories Inc., Westgrove, Pa.) for 1 hour at 37° C. The cells were washed again with in 0.1% dried skim milk powder dissolved in PBS and mounted in a solution composed of

50% glycerol and 50% PBS to which phenylenediamine (Sigma, St. Louis, Mo.) had been added to a final concentration of 1 mg/ml. The sample were analyzed using a Bio-Rad MRC 1000 confocal microscope with Laser Sharp software version 2.1T (Bio-Rad, Hercules, Calif.). Staining for occludin was assigned the pseudo-color red, whereas VE-cadherin staining was assigned pseudo-color green using Confocal Assistant 4.02 software. Immunofluorescence photographs of monolayer cultures of human aortic endothelial cells immunolabeled for occludin (red color) and VE-cadherin (green color) are shown in FIGS. **4**A and **4**B. Colocalization of occludin and VE-cadherin is indicated by the yellow color. Arrows indicate gaps between the cells. Note that the endothelial cells retract from one another when cultured in the presence of H-QYLYHYCVVD-OH (SEQ ID NO:2; FIG. **4**B), indicating that adhesion is decreased between the cells. Furthermore, the cells do not form cobblestone-like monolayers when exposed to this peptide. Also note that surface expression of both VE-cadherin and occludin is greatly reduced in the cells treated with H-QYLYHYCVVD-OH (SEQ ID NO:2), as compared to the VE-cadherin and occludin levels expressed by untreated cells.

EXAMPLE 3

Effect of Representative Modulating Agents on Vasopermeability

This Example illustrates a vasopermeability assay for evaluating the effects of occludin-modulating agents on endothelial cell permeability in vivo.

A. Miles Assay for Vascular Permeability

The ability of cyclic and linear peptides to increase vascular permeability was assessed utilizing the Miles assay (McClure et al., *J. Pharmacological & Toxicological Meth.* 32:49–521994). The peptides were dissolved in phosphate buffered saline (PBS) at a concentration of 100 μg/ml. Adult rats were given 100 μl subdermal injections of each peptide solution into their shaved backs, followed 15 minutes later by a single 250 μl injection of 1% Evans blue dissolved in PBS into their tail veins. The subdermal injection sites were visually monitored for the appearance of blue dye. Once the dye appeared (15 minutes after injection), each subdermal injection site was excised, weighed, and placed in 1 ml dimethylformamide for 24 hours to extract the dye. The optical density of the dye extracts was determined at 620 nm. The effects of injecting either phosphate buffered saline, phosphate buffered saline containing acetyl-QYLYHYCVVD-NH_2 (SEQ ID NO:2) H-QYLYHYCVVD-NH_2 (SEQ ID NO:2), or H-QYLYHYCVVD-OH (SEQ ID NO:2) into sites along the shaved back of a rat on the accumulation of Evans blue at the injection sites is shown in FIG. **5**. Note that more blue dye has accumulated at the sites where the peptide H-QYLYHYCVVD-OH (SEQ ID NO:2) was injected, as opposed to the sites where either phosphate buffered saline, phosphate buffered saline containing acetyl-QYLYHYCVVD-NH_2 (SEQ ID NO:2), or H-QYLYHYCVVD-NH_2 (SEQ ID NO:2) were injected.

FIG. **6** shows a histogram depicting the optical densities of dimethylformamide extracts prepared from the excised injection sites shown in FIG. **5**. Note that more dye was extracted from the sites injected with H-QYLYHYCVVD-OH (SEQ ID NO:2), than from sites injected with either phosphate buffered saline, acetyl-QYLYHYCVVD-NH_2 (SEQ ID NO:2), or H-QYLYHYCVVD-NH_2 (SEQ ID NO:2).

The effects of injecting either phosphate buffered saline, phosphate buffered saline containing acetyl-CLYHYC-NH_2 (SEQ ID NO:3) or H-CLYHYC-OH (SEQ ID NO:3) into sites along the shaved back of a rat on the accumulation of Evans blue at the injection sites is shown in FIG. **7**. FIG. **8** shows a histogram depicting the optical densities of dimethylformamide extracts prepared from the excised sites of the shaved back of a rat that received injections of either phosphate buffered saline, phosphate buffered saline containing acetyl-CLYHYC-NH_2 (SEQ ID NO:3), or H-CLYHYC-OH (SEQ ID NO:3) at a concentration of 100 μg/ml, followed 15 minutes later by a single injection of Evans blue into the tail vein. Note that more dye was extracted from the sites injected with H-CLYHYC-OH (SEQ ID NO:3), than from sites injected with either phosphate buffered saline, or acetyl-CLYHYC-NH_2 (SEQ ID NO:3).

EXAMPLE 4

Effect of Representative Modulating Agents on Electrical Resistance Across Cell Monolayer

This Example illustrates an electrical resistance assay for evaluating the effects of occludin-modulating agents on epithelial cell adhesion.

Madin Darby canine kidney (MDCK) cells were plated in Millicells (Millipore, Bedford, Mass.), at a density of 300,000 cells per Millicell, and cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma, St. Louis, Mo.) containing 5% fetal calf serum (Sigma, St. Louis, Mo.). Monolayers were exposed to the modulating agent dissolved in medium at a final concentration of 0.5 mg/ml for a period of 24 hours. The electrical resistance was measured using the EVOM device (World Precision Instruments, Sarasota, Fla.). At the time of measurement, fresh medium, with or without the modulating agent, may be added to the Millicells.

FIG. **9** is a histogram depicting the mean electrical resistance across MDCK cell monolayers cultured for 24 hours in medium alone (Control), or medium containing H-QYLYHYCVVD-NH_2 (SEQ ID NO:2; Peptide 2), H-QYLYHYCVVD-COOH (SEQ ID NO:2; Peptide 3) or N-Ac-CLYHYC-NH_2 (SEQ ID NO:3; Peptide 4) at a concentration of 0.5 mg/ml. Duplicate measurements were taken, and error bars represent the standard deviation. Peptide 2 was found to reduce the electrical resistance, while peptides 3 and 4 increased the electrical resistance across the monolayer, relative to the control. These results demonstrate the ability of occludin modulating agents to modulate the formation of tight junctions in epithelial cells. In particular, certain agents (such as peptides 3 and 4, above) stimulate the formation of tight junctions in epithelial cells.

From the foregoing, it will be evident that although specific embodiments of the invention have been described herein for the purpose of illustrating the invention, various modifications may be made without deviating from the spirit and scope of the invention.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 52

<210> SEQ ID NO 1
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Occludin cell adhesion recognition sequence

<400> SEQUENCE: 1

Leu Tyr His Tyr
1

<210> SEQ ID NO 2
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulating agent

<400> SEQUENCE: 2

Gln Tyr Leu Tyr His Tyr Cys Val Val Asp
1 5 10

<210> SEQ ID NO 3
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulating agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic peptide

<400> SEQUENCE: 3

Cys Leu Tyr His Tyr Cys
1 5

<210> SEQ ID NO 4
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: N-cadherin cell adhesion recognition sequence

<400> SEQUENCE: 4

Phe His Leu Arg Ala His Ala Val Asp Ile Asn Gly Asn Gln Val
1 5 10 15

<210> SEQ ID NO 5
<211> LENGTH: 48
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 5

Gly Val Asn Pro Thr Ala Gln Ser Ser Gly Ser Leu Tyr Gly Ser Gln
1 5 10 15

Ile Tyr Ala Leu Cys Asn Gln Phe Tyr Thr Pro Ala Ala Thr Gly Leu
20 25 30

Tyr Val Asp Gln Tyr Leu Tyr His Tyr Cys Val Val Asp Pro Gln Glu
35 40 45

-continued

<210> SEQ ID NO 6
<211> LENGTH: 48
<212> TYPE: PRT
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 6

Gly Val Asn Pro Thr Ala Gln Ala Ser Gly Ser Met Tyr Gly Ser Gln
1 5 10 15

Ile Tyr Met Ile Cys Asn Gln Phe Tyr Thr Pro Gly Gly Thr Gly Leu
20 25 30

Tyr Val Asp Gln Tyr Leu Tyr His Tyr Cys Val Val Asp Pro Gln Glu
35 40 45

<210> SEQ ID NO 7
<211> LENGTH: 48
<212> TYPE: PRT
<213> ORGANISM: Canis sp.

<400> SEQUENCE: 7

Gly Val Asn Pro Thr Ala Gln Ala Ser Gly Ser Leu Tyr Ser Ser Gln
1 5 10 15

Ile Tyr Ala Met Cys Asn Gln Phe Tyr Ala Ser Thr Ala Thr Gly Leu
20 25 30

Tyr Met Asp Gln Tyr Leu Tyr His Tyr Cys Val Val Asp Pro Gln Glu
35 40 45

<210> SEQ ID NO 8
<211> LENGTH: 50
<212> TYPE: PRT
<213> ORGANISM: dipodomys sp.

<400> SEQUENCE: 8

Gly Val Asn Pro Arg Ala Gly Leu Gly Ala Ser Ser Gly Ser Leu Tyr
1 5 10 15

Tyr Asn Gln Met Leu Met Leu Cys Asn Gln Met Met Ser Pro Val Ala
20 25 30

Gly Gly Ile Met Asn Gln Tyr Leu Tyr His Tyr Cys Met Val Asp Pro
35 40 45

Gln Glu
50

<210> SEQ ID NO 9
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Representative occludin cell adhesion recognition
sequence

<400> SEQUENCE: 9

Leu Tyr His Tyr Leu Tyr His Tyr
1 5

<210> SEQ ID NO 10
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Representative occludin cell adhesion recognition
sequence

-continued

<400> SEQUENCE: 10

Gln Leu Tyr His Tyr Gln Leu Tyr His Tyr Gln Leu Tyr His Tyr
1 5 10 15

<210> SEQ ID NO 11
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion recognition sequence bound by N-cell adhesion molecules

<400> SEQUENCE: 11

Lys Tyr Ser Phe Asn Tyr Asp Gly Ser Glu
1 5 10

<210> SEQ ID NO 12
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent

<400> SEQUENCE: 12

Tyr Leu Tyr His Tyr Cys Val Val Asp
1 5

<210> SEQ ID NO 13
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent

<400> SEQUENCE: 13

Leu Tyr His Tyr Cys Val Val Asp
1 5

<210> SEQ ID NO 14
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent

<400> SEQUENCE: 14

Gln Tyr Leu Tyr His Tyr Cys
1 5

<210> SEQ ID NO 15
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent

<400> SEQUENCE: 15

Tyr Leu Tyr His Tyr Cys
1 5

<210> SEQ ID NO 16
<211> LENGTH: 5

-continued

<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent

<400> SEQUENCE: 16

Leu Tyr His Tyr Cys
1 5

<210> SEQ ID NO 17
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent

<400> SEQUENCE: 17

Gln Tyr Leu Tyr His Tyr
1 5

<210> SEQ ID NO 18
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent

<400> SEQUENCE: 18

Tyr Leu Tyr His Tyr
1 5

<210> SEQ ID NO 19
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent

<400> SEQUENCE: 19

Cys Asp Gly Tyr Pro Lys Asp Cys Lys Gly
1 5 10

<210> SEQ ID NO 20
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic Peptide

<400> SEQUENCE: 20

Cys Asp Gly Tyr Pro Lys Asp Cys Lys Gly
1 5 10

<210> SEQ ID NO 21
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent

<400> SEQUENCE: 21

-continued

Cys Gly Asn Leu Ser Thr Cys Met Leu Gly
1 5 10

<210> SEQ ID NO 22
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic Peptide

<400> SEQUENCE: 22

Cys Gly Asn Leu Ser Thr Cys Met Leu Gly
1 5 10

<210> SEQ ID NO 23
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent

<400> SEQUENCE: 23

Cys Tyr Ile Gln Asn Cys Pro Leu Gly
1 5

<210> SEQ ID NO 24
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic peptide

<400> SEQUENCE: 24

Cys Tyr Ile Gln Asn Cys Pro Leu Gly
1 5

<210> SEQ ID NO 25
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic peptide

<400> SEQUENCE: 25

Cys Leu Tyr His Tyr Cys
1 5

<210> SEQ ID NO 26
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic peptide

<400> SEQUENCE: 26

-continued

Cys Leu Tyr His Tyr Cys
1 5

<210> SEQ ID NO 27
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic peptide

<400> SEQUENCE: 27

Cys Gln Tyr Leu Tyr His Tyr Cys
1 5

<210> SEQ ID NO 28
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic peptide

<400> SEQUENCE: 28

Cys Gln Tyr Leu Tyr His Tyr Cys
1 5

<210> SEQ ID NO 29
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic peptide

<400> SEQUENCE: 29

Cys Tyr Leu Tyr His Tyr Cys
1 5

<210> SEQ ID NO 30
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic peptide

<400> SEQUENCE: 30

Cys Tyr Leu Tyr His Tyr Cys
1 5

<210> SEQ ID NO 31
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic peptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES

-continued

<222> LOCATION: (6)
<223> OTHER INFORMATION: Where Xaa is beta,beta-dimethylcysteine

<400> SEQUENCE: 31

Cys Leu Tyr His Tyr Xaa
1 5

<210> SEQ ID NO 32
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic Peptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)
<223> OTHER INFORMATION: Where Xaa is beta,beta-tetramethylene cysteine

<400> SEQUENCE: 32

Xaa Leu Tyr His Tyr Cys
1 5

<210> SEQ ID NO 33
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic peptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)
<223> OTHER INFORMATION: beta,beta-pentamethylene cysteine

<400> SEQUENCE: 33

Xaa Leu Tyr His Tyr Cys
1 5

<210> SEQ ID NO 34
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic peptide
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)
<223> OTHER INFORMATION: Where Xaa is beta-mercaptopropionic acid

<400> SEQUENCE: 34

Xaa Leu Tyr His Tyr Cys
1 5

<210> SEQ ID NO 35
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic peptide
<220> FEATURE:

-continued

<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)
<223> OTHER INFORMATION: beta,beta-pentamethylene-beta-mercaptopropionic acid

<400> SEQUENCE: 35

Xaa Leu Tyr His Tyr Cys
1 5

<210> SEQ ID NO 36
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic peptide

<400> SEQUENCE: 36

Lys Leu Tyr His Tyr Asp
1 5

<210> SEQ ID NO 37
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic peptide

<400> SEQUENCE: 37

Lys Gln Tyr Leu Tyr His Tyr Asp
1 5

<210> SEQ ID NO 38
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulation agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic peptide

<400> SEQUENCE: 38

Trp Gly Gly Trp
1

<210> SEQ ID NO 39
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: E-cadherin cell adhesion recognition sequence

<400> SEQUENCE: 39

Leu Phe Ser His Ala Val Ser Ser Asn Gly
1 5 10

<210> SEQ ID NO 40
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell

-continued

adhesion modulating agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic peptide

<400> SEQUENCE: 40

Cys Tyr Leu Tyr His Tyr Cys
1 5

<210> SEQ ID NO 41
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulating agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic peptide

<400> SEQUENCE: 41

Cys Gln Tyr Leu Tyr His Tyr Cys
1 5

<210> SEQ ID NO 42
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulating agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic Peptide

<400> SEQUENCE: 42

Lys Gln Tyr Leu Tyr His Tyr Asp
1 5

<210> SEQ ID NO 43
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulating agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic peptide

<400> SEQUENCE: 43

Tyr Leu Tyr His Tyr
1 5

<210> SEQ ID NO 44
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulating agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic peptide

<400> SEQUENCE: 44

Gln Tyr Leu Tyr His Tyr
1 5

<210> SEQ ID NO 45
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

-continued

<223> OTHER INFORMATION: Description of Artificial Sequence: Cell adhesion modulating agent
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic peptide

<400> SEQUENCE: 45

Lys Leu Tyr His Tyr Asp
1 5

<210> SEQ ID NO 46
<211> LENGTH: 51
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (8)
<223> OTHER INFORMATION: Where Xaa is any amino acid residue
<220> FEATURE:
<221> NAME/KEY: MOD RES
<222> LOCATION: (17)
<223> OTHER INFORMATION: Where Xaa is any amino acid residue
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (22)..(23)
<223> OTHER INFORMATION: Where Xaa is any amino acid residue
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (29)
<223> OTHER INFORMATION: Where Xaa is any amino acid residue
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (31)
<223> OTHER INFORMATION: Where Xaa is any amino acid residue
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (37)
<223> OTHER INFORMATION: Where Xaa is any amino acid residue

<400> SEQUENCE: 46

Gly Val Asn Pro Thr Ala Gln Xaa Gly Ala Ser Ser Gly Ser Leu Tyr
1 5 10 15

Xaa Ser Gln Ile Tyr Xaa Xaa Cys Asn Gln Phe Tyr Xaa Pro Xaa Ala
20 25 30

Thr Gly Leu Tyr Xaa Asp Gln Tyr Leu Tyr His Tyr Cys Val Val Asp
35 40 45

Pro Gln Glu
50

<210> SEQ ID NO 47
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Claudin cell adhesion recognition sequence
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (2)
<223> OTHER INFORMATION: Where Xaa is either Lysine or Arginine
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (3)
<223> OTHER INFORMATION: Where Xaa is an independently selected amino acid residue
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (4)
<223> OTHER INFORMATION: Where Xaa is an independently selected amino acid residue
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (5)

-continued

<223> OTHER INFORMATION: Where Xaa is either Serine or Arginine
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)
<223> OTHER INFORMATION: Where Xaa is either tyrosine or phenylalanine
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (7)
<223> OTHER INFORMATION: Where Xaa is an independently selected amino acid residue

<400> SEQUENCE: 47

Trp Xaa Xaa Xaa Xaa Xaa Xaa Gly
1 5

<210> SEQ ID NO 48
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Non-classical cell adhesion recognition sequence
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)
<223> OTHER INFORMATION: Where Xaa is an independently selected amino acid residue
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (3)
<223> OTHER INFORMATION: Where Xaa is an independently selected amino acid residue
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (4)
<223> OTHER INFORMATION: Where Xaa is isoleucine, leucine or valine
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (5)
<223> OTHER INFORMATION: Where Xaa is aspartic acid, asparigine or glutamic acid
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (6)
<223> OTHER INFORMATION: Where Xaa is an independently selected amino acid residue
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (7)
<223> OTHER INFORMATION: Where Xaa is and idependently selected amino acid residue
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (8)
<223> OTHER INFORMATION: Where Xaa is serine, threonin or asparigine

<400> SEQUENCE: 48

Xaa Phe Xaa Xaa Xaa Xaa Xaa Xaa Gly
1 5

<210> SEQ ID NO 49
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Representative claudin cell adhesion recognition sequence

<400> SEQUENCE: 49

Ile Tyr Ser Tyr
1

-continued

<210> SEQ ID NO 50
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Representative claudin cell adhesion recognition
sequence

<400> SEQUENCE: 50

Thr Ser Ser Tyr
1

<210> SEQ ID NO 51
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Representative claudin cell adhesion recognition
sequence

<400> SEQUENCE: 51

Val Thr Ala Phe
1

<210> SEQ ID NO 52
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Representative claudin cell adhesion recognition
sequence

<400> SEQUENCE: 52

Val Ser Ala Phe

What is claimed is:

1. A method for identifying a compound capable of modulating occludin-mediated cell adhesion, comprising:

(a) contacting a test compound with an antibody or antigen-binding fragment thereof that binds to a modulating agent comprising the sequence LYHY (SEQ ID NO:1), wherein the agent comprises no more than 30 consecutive amino acid residues present within an occludin molecule; and

(b) detecting the level of antibody that binds to the test compound, and therefrom identifying a compound capable of modulating occludin-mediated cell adhesion.

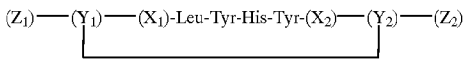
2. A method according to claim 1, wherein the agent is a linear peptide.

3. A method according to claim 2, wherein the agent comprises 5–16 consecutive amino acid residues present within an occludin.

4. A method according to claim 3, wherein the agent comprises a sequence selected from the group consisting of QYLYHYCVVD (SEQ ID NO:2), YLYHYCVVD (SEQ ID NO:12), LYHYCVVD (SEQ ID NO:13), QYLYHYC (SEQ ID NO:14), YLYHYC (SEQ ID NO:15), LYHYC (SEQ ID NO:16), QYLYHY (SEQ ID NO:17) and YLYHY (SEQ ID NO:18).

5. A method according to claim 1, wherein the agent is a cyclic peptide.

6. A method according to claim 5, wherein the cyclic peptide has the formula:



wherein X_1, and X_2 are optional, and if present, are independently selected from the group consisting of amino acid residues and combinations thereof in which the residues are linked by peptide bonds, and wherein X_1 and X_2 independently range in size from 0 to 10 residues, such that the sum of residues contained within X_1 and X_2 ranges from 1 to 12;

wherein Y_1 and Y_2 are independently selected from the group consisting of amino acid residues, and wherein a covalent bond is formed between residues Y_1 and Y_2; and

wherein Z_1 and Z_2 are optional, and if present, are independently selected from the group consisting of amino acid residues and combinations thereof in which the residues are linked by peptide bonds.

7. A method according to claim 6, wherein the cyclic peptide is selected from the group consisting of CLYHYC (SEQ ID NO:3), CYLYHYC (SEQ ID NO:40), CQYLYHYC(SEQ ID NO:41), KQYLYHYD (SEQ ID NO:42), YLYHY(SEQ ID NO:43), QYLYHY (SEQ ID NO:44) and KLYHYD (SEQ ID NO:45) and derivatives of

the foregoing sequences having one or more C-terminal and/or N-terminal modifications.

8. A method according to claim **1**, wherein the step of detecting is performed using an ELISA.

9. A method according to claim **1**, wherein the test compound is produced by an expression library.

* * * * *

US006719902B1

(12) **United States Patent**
Alvarez et al.

(10) **Patent No.: US 6,719,902 B1**
(45) **Date of Patent: Apr. 13, 2004**

(54) **FE(O)-BASED BIOREMEDIATION OF AQUIFERS CONTAMINATED WITH MIXED WASTES**

(75) Inventors: **Pedro J. Alvarez**, Iowa City, IA (US); **Brian A. Till**, Milwaukee, WI (US); **Lenly J Weathers**, Cookeville, TN (US); **Gene F. Parkin**, Iowa City, IA (US); **Jerald L. Schnoor**, Iowa City, IA (US)

(73) Assignee: **The University of Iowa Research Foundation**, Iowa City, IA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/446,581**

(22) PCT Filed: **Apr. 24, 1998**

(86) PCT No.: **PCT/US98/08196**

§ 371 (c)(1),
(2), (4) Date: **Aug. 27, 2001**

(87) PCT Pub. No.: **WO98/49106**

PCT Pub. Date: **Nov. 5, 1998**

Related U.S. Application Data

(60) Provisional application No. 60/044,810, filed on Apr. 25, 1997.

(51) **Int. Cl.**[7] **C02F 3/34**; C02F 3/28

(52) **U.S. Cl.** **210/601**; 210/617; 210/631; 210/170; 210/903; 210/909; 210/913; 210/914; 435/176; 435/177; 405/128.45

(58) **Field of Search** 210/601, 170, 210/615–617, 631, 747, 150, 151, 903, 909, 912–914; 435/262, 262.5, 176, 177, 180; 405/128.15, 128.45

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,493,895	A	* 1/1985	Colaruotolo et al.	435/262
4,554,101	A	11/1985	Hopp	
4,566,469	A	* 1/1986	Semp et al.	131/308
4,839,052	A	* 6/1989	Maree	210/603
5,266,213	A	11/1993	Gillham	210/747
5,318,699	A	6/1994	Robertson et al.	210/151
5,342,769	A	* 8/1994	Hunter et al.	435/166
5,362,394	A	11/1994	Blowes et al.	210/617

(List continued on next page.)

FOREIGN PATENT DOCUMENTS

EP	0522946	1/1993
EP	0595441	5/1994
JP	52130150	11/1977
JP	52-130150	* 11/1977
JP	02/119992	5/1990
JP	2-119992	* 5/1990

OTHER PUBLICATIONS

Aguilar et al., "Degradation of volatile fatty acids by differently enriched methanogenic cultues: Kinetics and inhibition," *Water Res.*, 29(2):505–509, 1995.

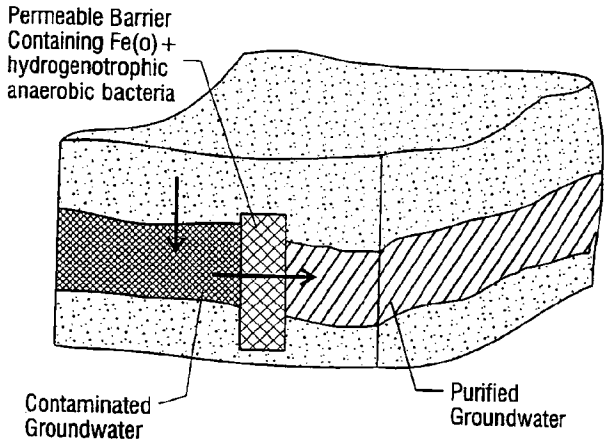
(List continued on next page.)

Primary Examiner—Fred G. Prince
(74) *Attorney, Agent, or Firm*—Fulbright & Jaworski, LLP

(57) **ABSTRACT**

Disclosed are methods, devices and apparatus for bioremediation of mixed waste aquifers, based on a synergistic combination of reductive treatment using zero-valent iron and anaerobic biotransformations. Also disclosed are methods for in situ and ex situ remediation of groundwater and wastewater via these iron-bacterial compositions in a variety of devices including batch reactors, permeable and semipermeable reactive barriers, flow-through reactors, fluidized bed reactors, and sediment tanks.

41 Claims, 16 Drawing Sheets



U.S. PATENT DOCUMENTS

5,415,777	A	*	5/1995	Krempen et al.	435/262.5
5,510,033	A	*	4/1996	Ensley et al.	210/611
5,512,479	A	*	4/1996	Steffan	435/262.5
5,514,279	A		5/1996	Blowes et al.	210/617
5,534,154	A		7/1996	Gillham	210/668
5,587,317	A	*	12/1996	Odom	435/262
5,618,427	A	*	4/1997	Seech et al.	210/601
5,679,568	A	*	10/1997	Imamura et al.	435/262.5
5,733,067	A	*	3/1998	Hunt et al.	405/128.5
5,789,649	A	*	8/1998	Batchelor et al.	588/206
5,833,855	A	*	11/1998	Saunders	210/611
5,908,267	A	*	6/1999	Schuring et al.	405/128.65

OTHER PUBLICATIONS

Assaf–Anid et al.,"Reductive dechlorination of carbon tetrachloride by cobalmin(II) in the presence of dithiothreitol: Mechanistic study, effect of redox potential and pH," *Environ. Sci. Technol.*, 28(2):246–250, 1994.

Belay and Daniels, "Elemental metals as electron sources for biological methane formation from carbon dioxide," *Antonie van Leeuvenhoek*, 57(1):1–8, 1990.

Blowes et al., "Passive remediation of groundwater using in situ treatment curtains," *In: Geoenvironment 2000: Characterization, Containment, Remediation, and Performance in Environmental Geotechnics*, Yalcin BA, Daniel DE, eds., Geotechnical Special Publication No. 46, American Society of Civil Engineers, New York, 2:1608–1621, 1995.

Chiu and Reinhard, "Metallocoenzyme–mediated reductive transformation of carbon tetrachloride in titanium (III) citrate aqueous solution," *Environ. Sci. Technol.*, 29(3):595–603, 1995.

Chiu and Reinhard, "Transformation of carbon tetrachloride by reduced vitamin B_{12} in aqueous cysteine solution," *Environ. Sci. Technol.*, 30:1882–1889, 1996.

Daniels et al., "Bacterial methanogenesis and growth from CO_2 with elemental iron as the sole source of electrons," *Science*, 237:509–511, 1987.

Doong and Wu, "Reductive dechlorination of chlorinated hydrocarbons in aqueous solutions containing ferrous and sulfide ions," *Chemosphere*, 24(8):1063–1075, 1992.

Freedman and Gossett, "Biodegradation of dichloromethane and its utilization as a growth substate under methanogenic conditions," *Appl. Environ. Microbiol.*, 57(10):2847–2857, 1991.

Gantzer and Wackett, "Reductive dechlorination catalyzed of bacterial transition–metal coenzymes," *Environ. Sci. Technol.*, 25(4):715–722, 1991.

Gillham and O'Hannesin, "Enhanced degradation of halogenated aliphatics by zero–valent iron," *Groundwater*, 32(6):958–967, 1994.

Gould, "The kinetics of hexavalent chromium reduction by metallic iron," *Water Res.*, 26:871–877, 1992.

Hansen et al., "Abiotic nitrate reduction to ammonium: Key role of green rust," *Envir. Sci. Technol.*, 30(6):2053–2056, 1996.

Helland et al., "Reductive dechlorination of carbon tetrachloride with elemental iron," *J. Hazard Mater.*, 41:205–216, 1995.

Holliger et al., "A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethylene to growth," *Appl. Environ. Microbiol.*, 59:2991–2997, 1993.

Johnson et al., "Kinetics of halogenated organic compound degradation by iron metal." *Environ. Sci. Technol.*, 30:2634–2640, 1996.

Lewis, "Factors influencing the stability and properties of green rusts," *Advances in GeoEcology*, 30:345–372, 1997.

Matheson and Tratnyek, "Reductive dehalogenation of chlorinated methanes by iron metal," *Environ. Sci. Technol.*, 28:2045–2053, 1994.

Matheson and Tratnyek, "Abiotic and biotic processes affecting the dechlorination of chlorinated solvents by zero–valent iron," *Abstracts of Papers*, 20th ACS National Meeting, American Chemical Society, Mar. 13–17, 1994, Abstract No. 135.

Morrison et al., "Coupled Reaction/Transport Modeling of a Chemoical Barrier for Controlling Uranium(VI) contamination in Groundwater," *J. Contaminant Hydrology*, 17:343–363, 1995.

Myneni et al., "Abiotic selenium redox transformations in the presence of Fe(II,III) oxides," *Science*, 278:1106–1109, 1997.

O'Hannesin and Gillham, "In situ degredation of halogenated organics by permeable reaction wall," *EPA Groundwater Currents*, 542:1–2, 1993.

Olowe et al., "La présence simultanée de rouille verte 2 et de bactéries sulfato–réductrices en corrosion perforante de palplanches en zone portuaire," *C.R. Acad. Sci. Paris*, t. 314, Série II, p1. 1157–1163, 1992.

Orth and Gillham, "Dechlorination of trichloroethylene in aqueous solution using Fe^0," *Environ. Sci. Technol.*, 30:66–71, 1996.

Perkins et al., "Effects of electron donors and inhibitors on reductive dechlorination of 2,4,6–trichlorophenol," *Water Res.*, 28(10):2101–2107, 1994.

Powell et al., "Coupled iron corrosion and chromate reduction: mechanisms for subsurface remediation," *Environ.Sci. Technol.*, 29:1913–1922, 1995.

Powell & Puls,. "Proton Generation by Dissolution of Intrinsic or Augmented Aluminosilicate Ninerals for In Situ Contaminant Generation by Zero–Valence–State Iron," *Environ. Sci. Technol. 1997, 31*(8), 2224–2251.

Reardon, "Anaerobic corrosion of granular iron: measurement and interpretation of hydrogen evolution rates," *J. Environ. Sci. Technol.*, 29:2936–2945, 1996.

Roberts et al., "Reductive elimination of chlorinated ethylenes by zero–valent metals," *Environ. Sci. Technol.*, 30(8):2654–2659, 1996.

Stromeyer et al., "Dichloromethane utilized by an anaerobic mixed culture: acetogenesis and methanogenesis," Abstract, *Biodegradation*, 2(2):129–137, 1991.

Sweeny, "The reductive treatment of industrial wastewaters. II process applications," *AIChE Symp. Ser.*, 77:72–78, 1980.

Tatara et al., "Effects of medium and trace metals on kinetics of carbon tetrachloride transformation by Psudomonas sp. strain KC," *Appl. Environ. Microbiol.*, 59(7):2126–2131, 1993.

Thiel, "The effect of methane analogues on methanogenesis in anaerobic digestion," *Water Res.*, 3:215–223, 1969.

Till et al., "Fe(0)–supported autotrophic denitrification," *Environ. Sci. Technol.*, 32(5), 634–639, 1998.

Trolard et al., "Mise en évidence d'un constituant de type <<rouilles vertes <<dans les sols hydromorphes. Proposition de l'existence d'un nouveau minéral: la <<fougérite>>," Trolard, Abdelmoula, Bourrié, Humbert and Génin, *C.R. Acad. Sci. Paris*, t. 323, série II a, p. 1015–1022, 1996.

Vidic and Pohland, "Treatment Walls", *Groundwater Remediation Technologies Analysis Center*, Technology Evaluation Report TE–96–01. Uiniversity of Pittsburgh, Pittsburgh, PA, Oct. 1996.

Weathers et al., "Chloroform cometabolism by a methanogenic consortium utilizing zero–valent iron as ultimate electron donor," *Abst. Gen. Meet. Am. Soc. Microbiol.*, 95(0):399, 1995.

Weathers, "Biological and metallic iron–promoted transformations of carbon tetrachloride and chloraform under methanogenic conditions," Ph.D. Dissertation, University of Iowa, 1995.

Weathers et al., "Methanogens couple anaerobic Fe(0) oxidation and $CHCl_3$ reduction," *Abst. Papers Am. Chem. Soc..*, 209(1–2):ENVR225, 1995.

Wild et al., "Anaerobic dechlorination of trichloroethene, tetrachloroethene and 1,2–dichloroethane by an acetogenic mixed culture in a fixed–bed reactor," Abstract, *Biodegradation*, 6(4):309–318, 1995.

Wong and Neumann, "Electric field mediated gene transfer," *Biochim. Biophys. Res. Commun.*, 107(2):584–587, 1982.

Yamamoto et al., "Kinetics and modeling of hexavalent chromium reduction in *Enterobacter cloacae*," *Biotechnol. Bioeng.*, 41:129–133, 1993.

Yang and Speece, "The effects of chloroform toxicity on methane fermentation," *Water Res.*, 20(10):1273–1280, 1986.

Holliger, "The anaerobic microbiology and biotreatment of chlorinated ethenes," *Curr. Opin. Biotechnol.*, 6:347–351, 1995.

Kaplan et al., "Formation of a barrier to groundwater contaminants by injection of zero–valent iron colloids: suspension properties," *Proc. In Situ Remediation: Scientific Basis for Current and Future Technologies Symposium. Thirty–third Hanford Symposium on Health and the Environment*; Nov. 7–11, 1994, Abstract and Table of contents from the Battelle website: www.battelle.org (Dec. 11, 2001).

MSE Technology Applications. "Analysis of Technologies for the Emplacement and Performance Assessment of Subsurface Reactive Barriers for DNAPL Containment". Report for U.S. Department of Energy (TTP# PEI–6–PL–341) under Contract No. DE–AC22–88ID12735, 1996. Abstract from US Dept. of Energy website (Nov. 1, 2001).

National Research Council, "Alternatives for groundwater cleanup," *Report of the National Academy of Science Committee on Groundwater Cleanup Alternatives*, National Academy Press, Washington, DC, 1994.

Zhang et al., "Enhancement of Fe(III), Co(III), and Cr(VI) reduction at elevated temperatures and by a thermophilic bacterium," *Appl. Biochem. Biotech.*, 57/58:923–932, 1996.

* cited by examiner

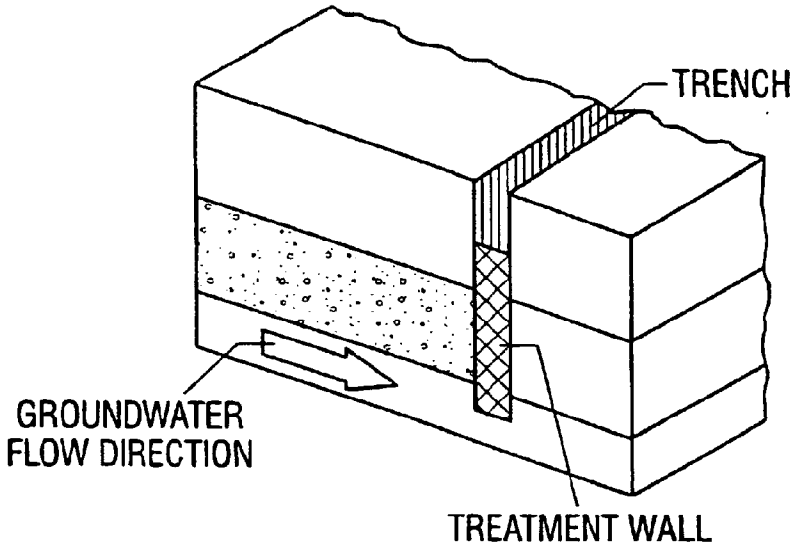

TRENCH
GROUNDWATER
FLOW DIRECTION
TREATMENT WALL

FIG. 1

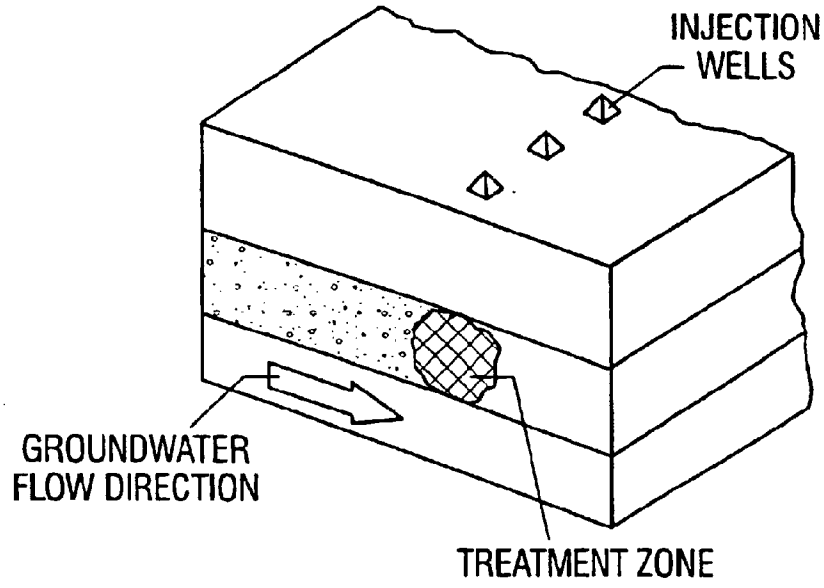

INJECTION
WELLS
GROUNDWATER
FLOW DIRECTION
TREATMENT ZONE

FIG. 2

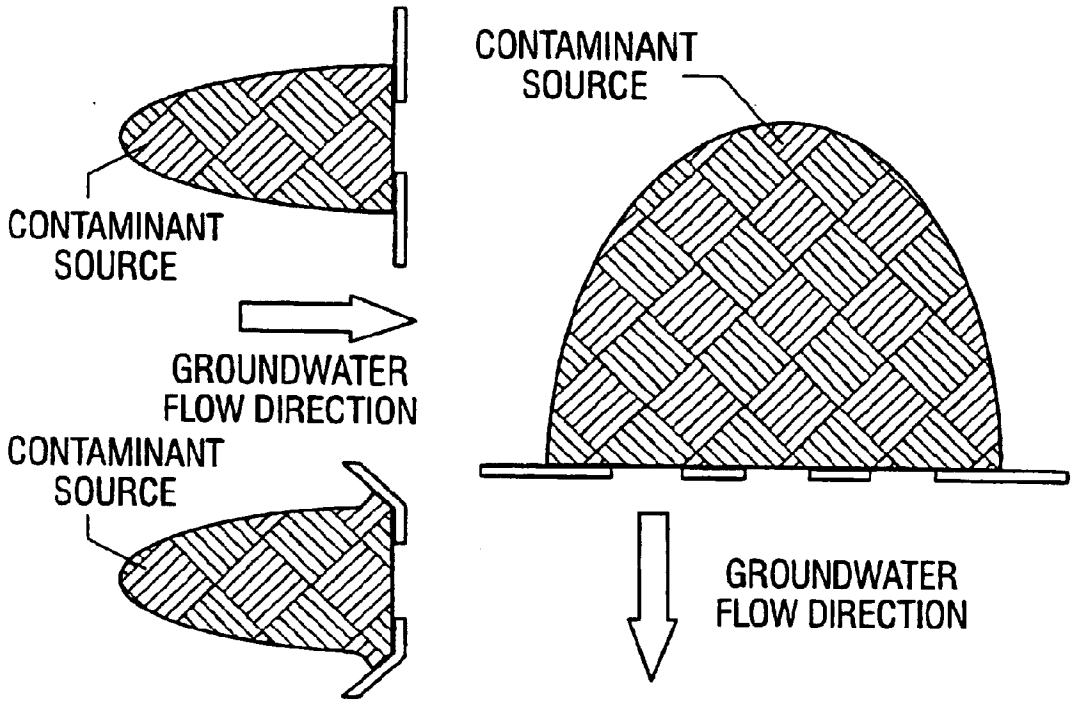

CONTAMINANT SOURCE
CONTAMINANT SOURCE
GROUNDWATER FLOW DIRECTION
CONTAMINANT SOURCE
GROUNDWATER FLOW DIRECTION

FIG. 3

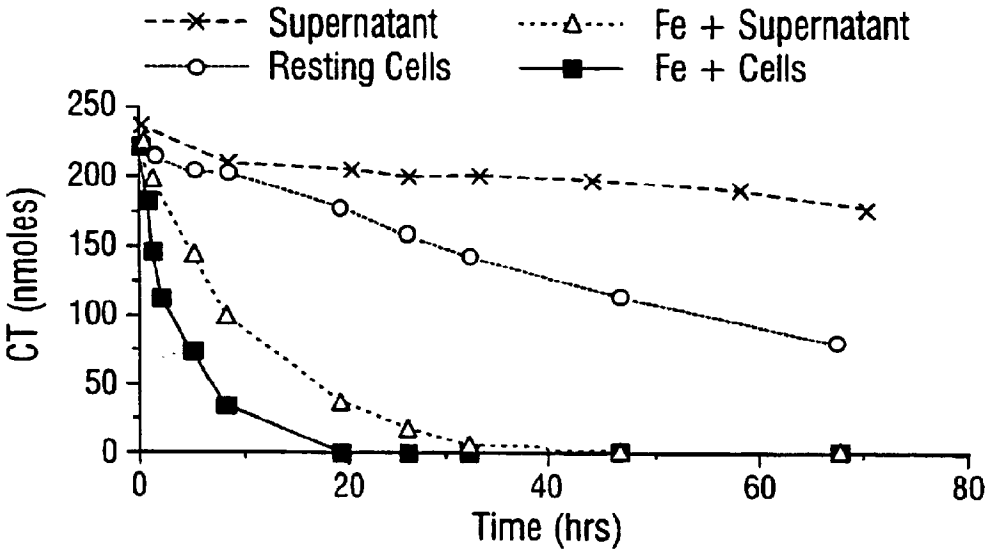

Supernatant
Fe + Supernatant
Resting Cells
Fe + Cells
CT (nmoles)
250
200
150
100
50
0
0
20
40
60
80
Time (hrs)

FIG. 4A

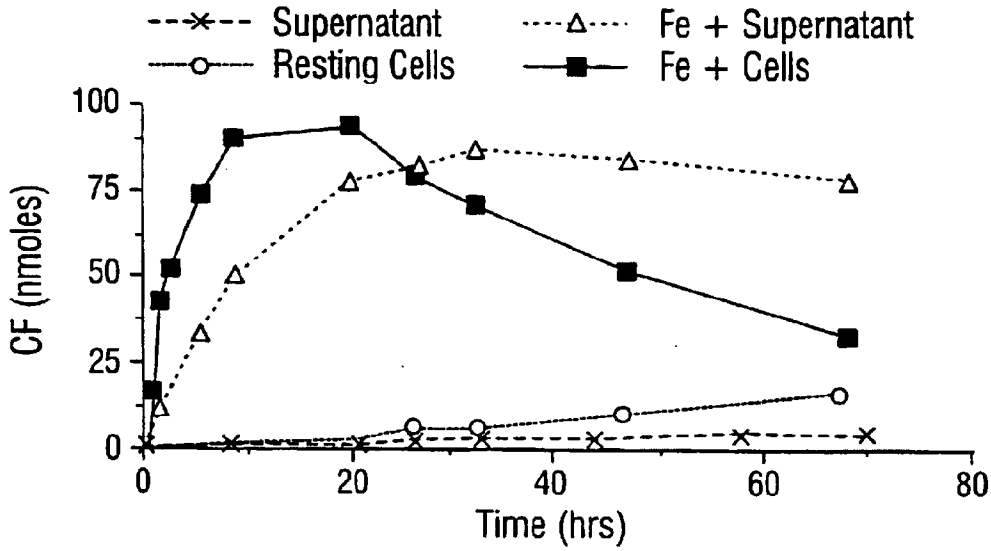

Supernatant
Fe + Supernatant
Resting Cells
Fe + Cells
CF (nmoles)
100
75
50
25
0
0
20
40
60
80
Time (hrs)

FIG. 4B

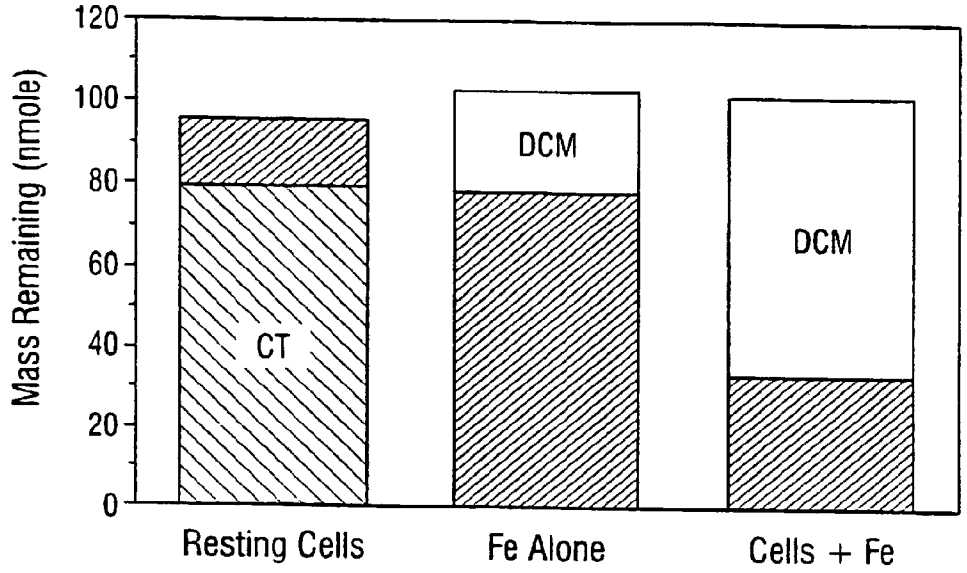

120
100
80
60
40
20
0
Mass Remaining (nmole)
CT
DCM
DCM
Resting Cells
Fe Alone
Cells + Fe

FIG. 5

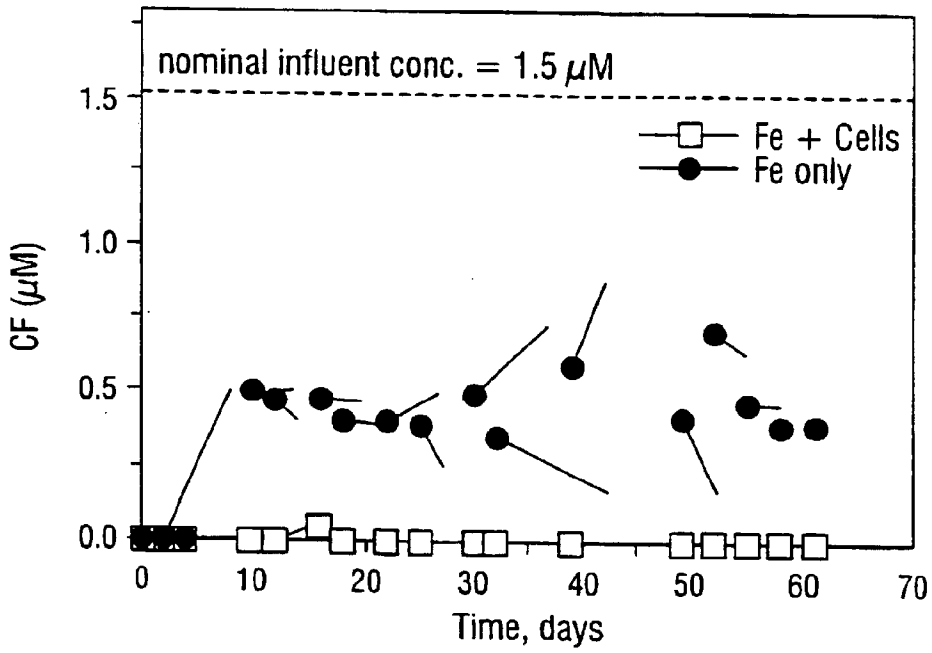

nominal influent conc. = 1.5 μM
Fe + Cells
Fe only
1.5
1.0
0.5
0.0
CF (μM)
0
10
20
30
40
50
60
70
Time, days

FIG. 6

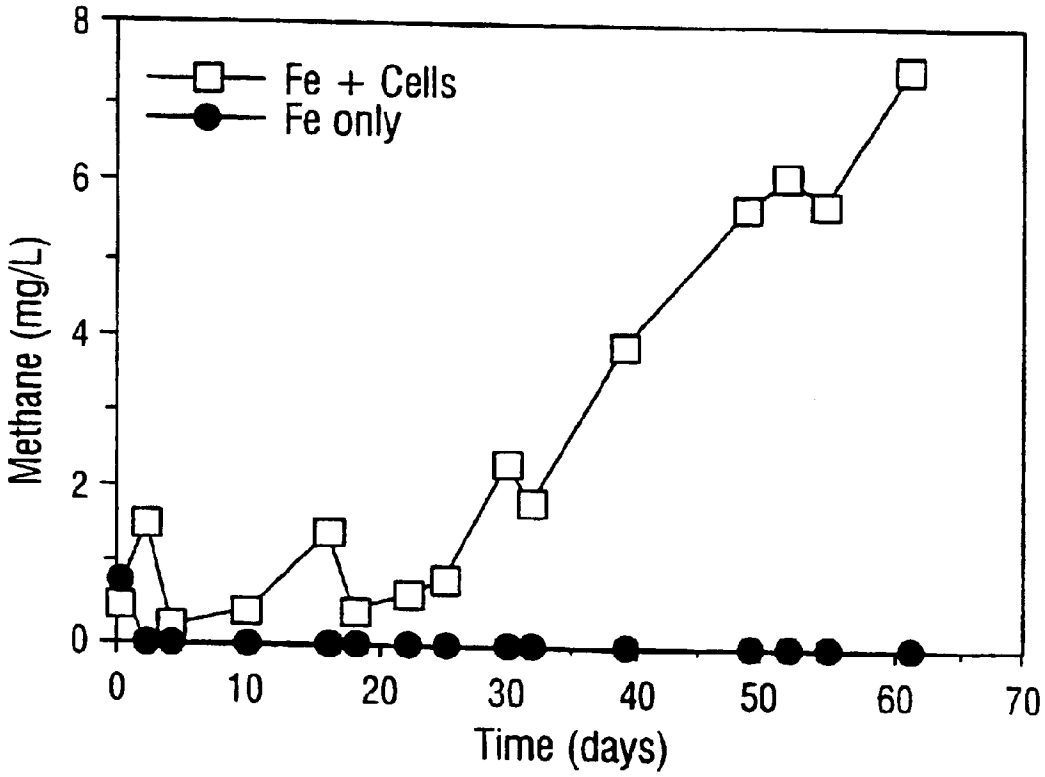

Fe + Cells
Fe only
Methane (mg/L)
Time (days)
0
2
4
6
8
0
10
20
30
40
50
60
70

FIG. 7

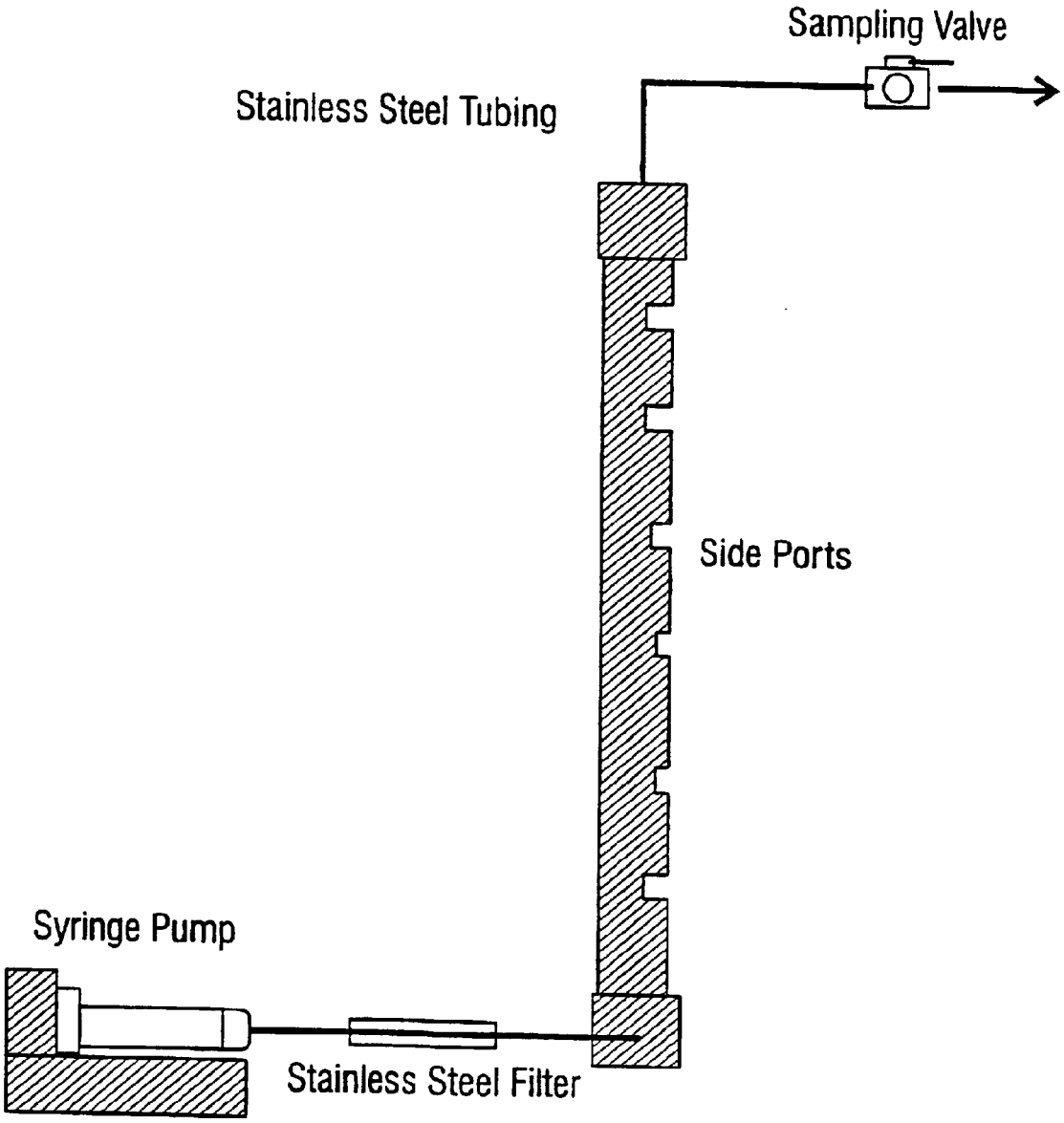

Sampling Valve
Stainless Steel Tubing
Side Ports
Syringe Pump
Stainless Steel Filter

FIG. 8

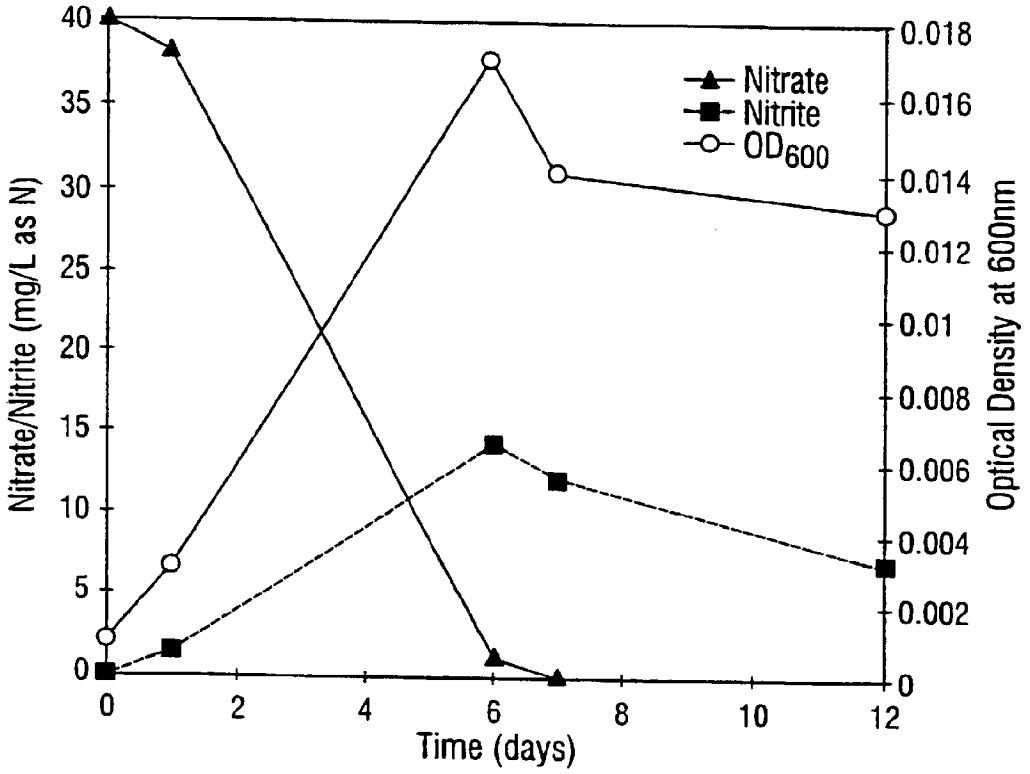

Nitrate
Nitrite
OD600
Nitrate/Nitrite (mg/L as N)
Optical Density at 600nm
Time (days)
0
5
10
15
20
25
30
35
40
0
0.002
0.004
0.006
0.008
0.01
0.012
0.014
0.016
0.018
0
2
4
6
8
10
12

FIG. 9A

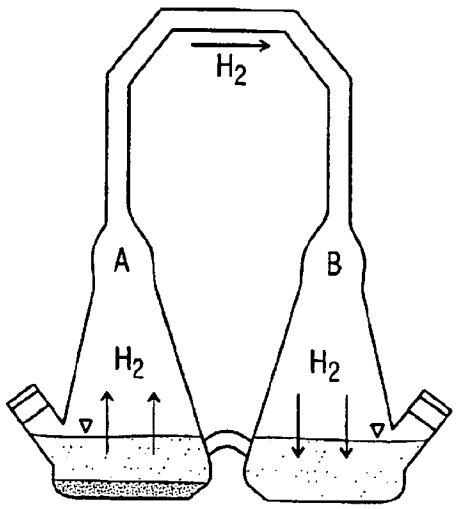

H2
A
B
H2
H2

FIG. 9B

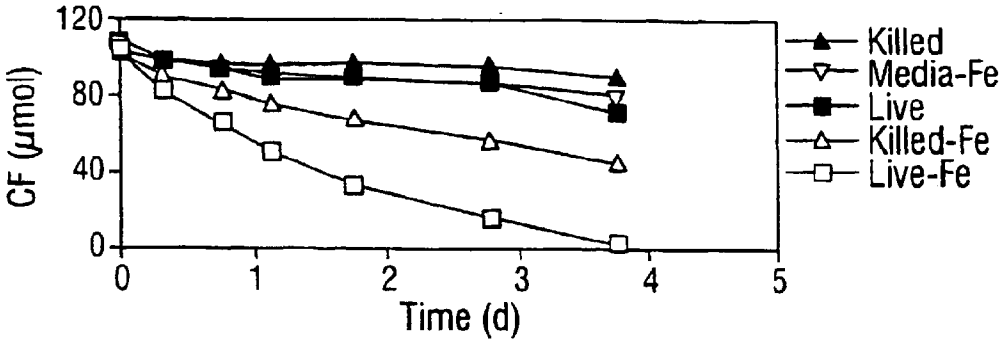


FIG. 10A

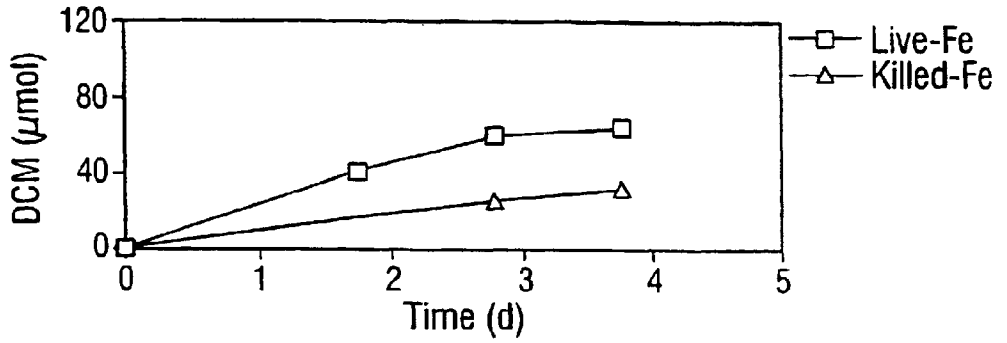


FIG. 10B

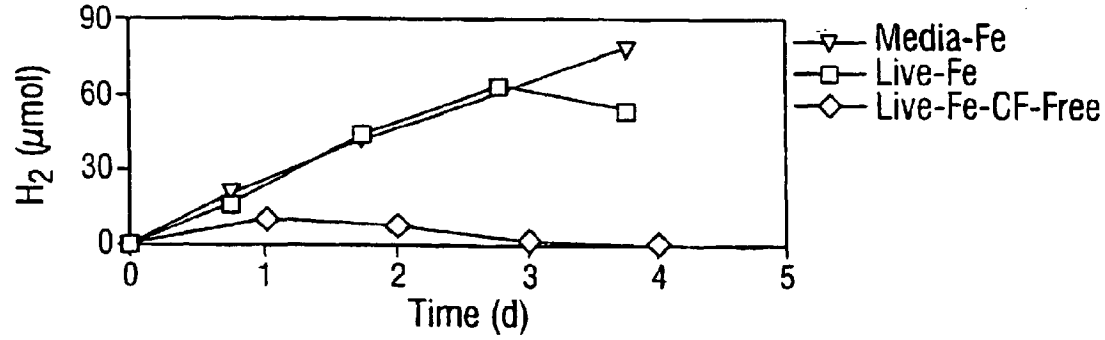


FIG. 10C

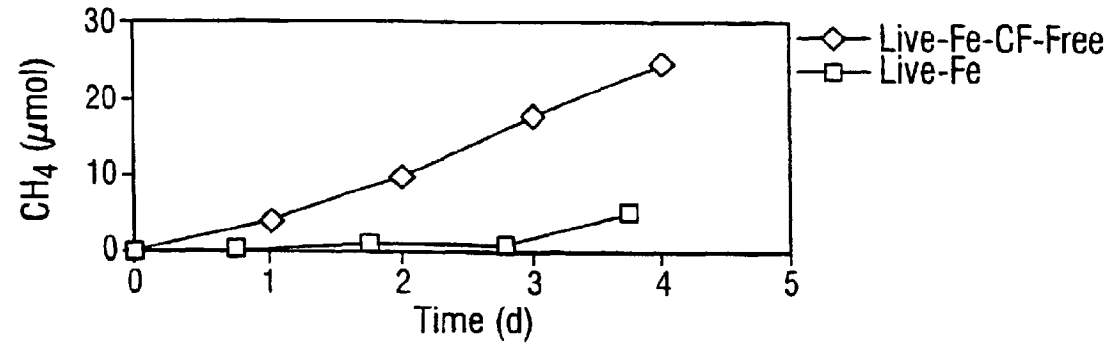


FIG. 10D

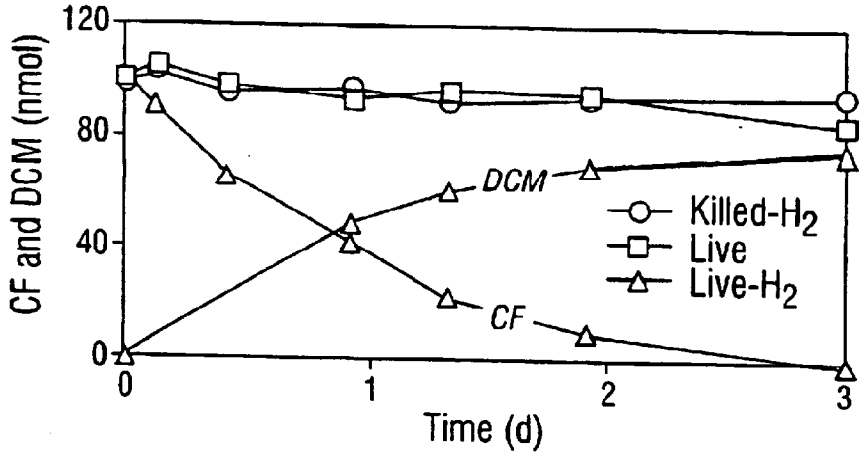


FIG. 11

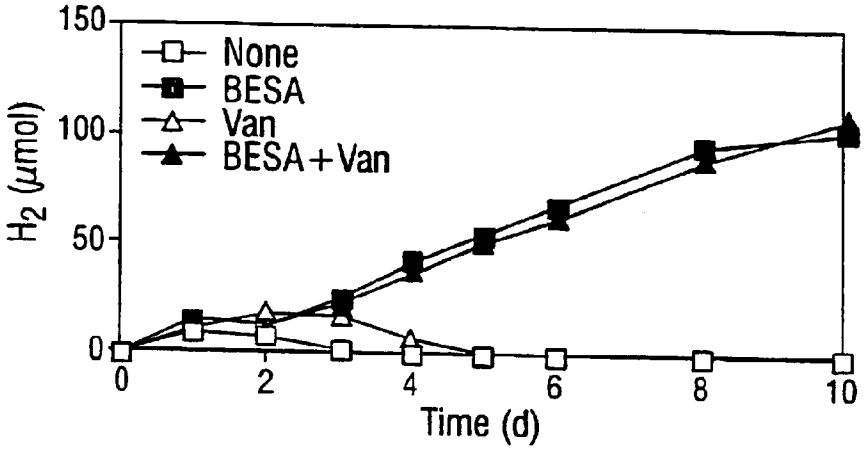


FIG. 12A

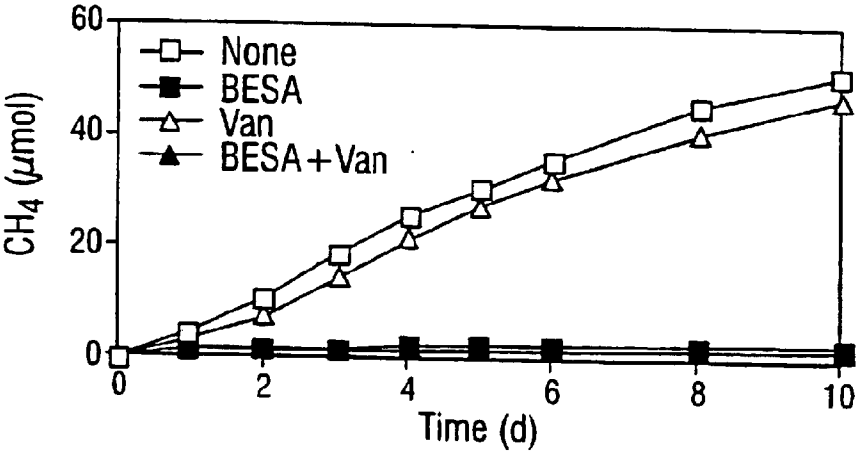


FIG. 12B

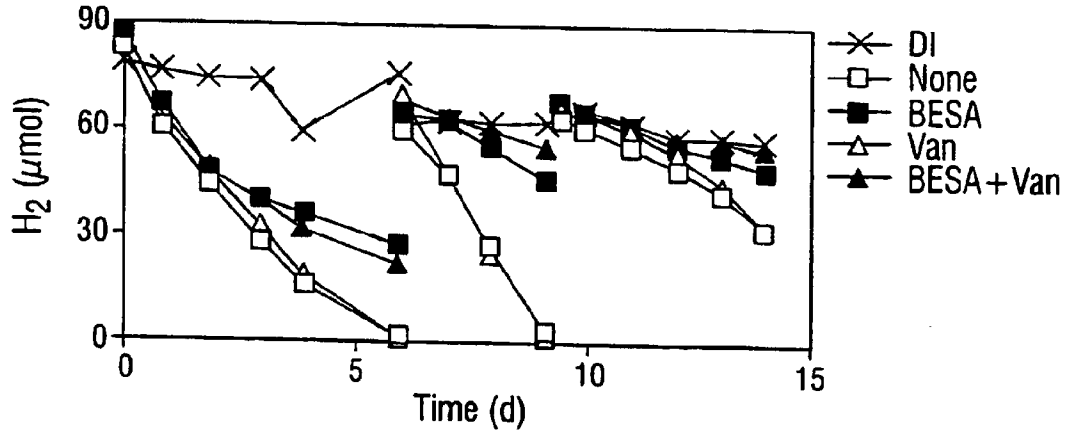


FIG. 13A

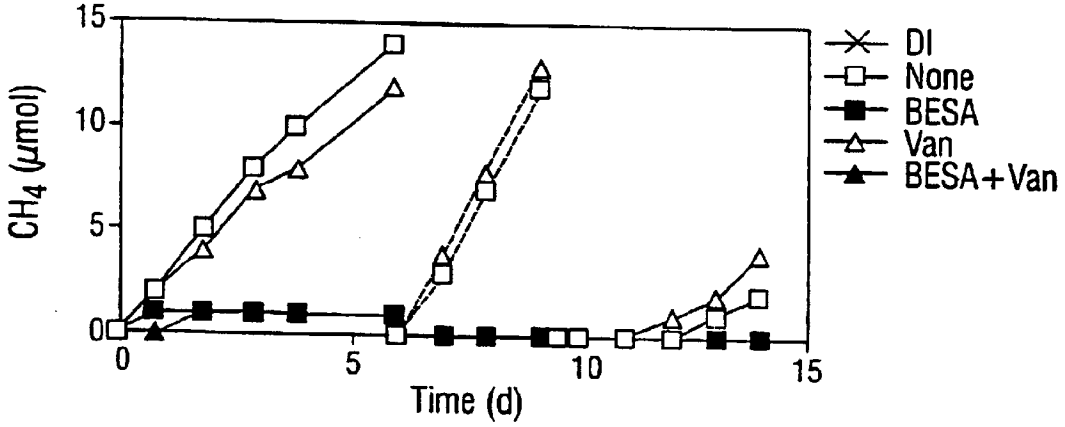


FIG. 13B

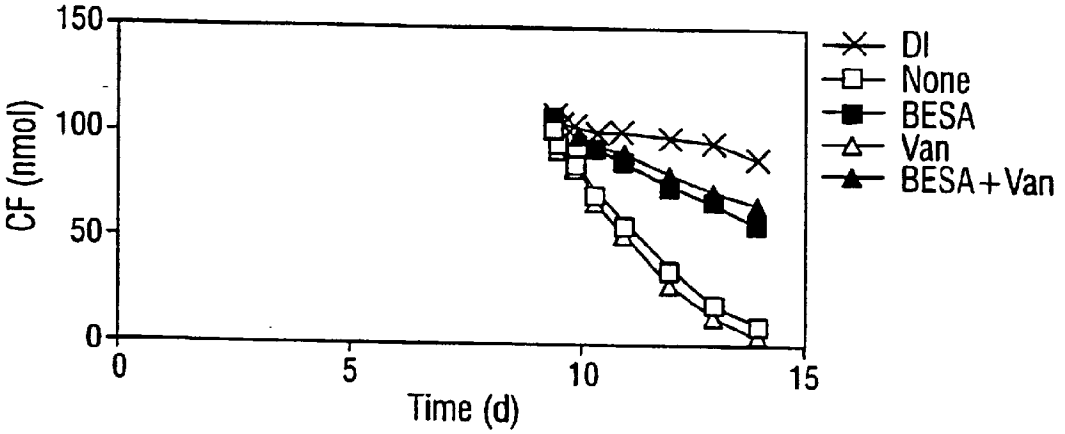


FIG. 13C

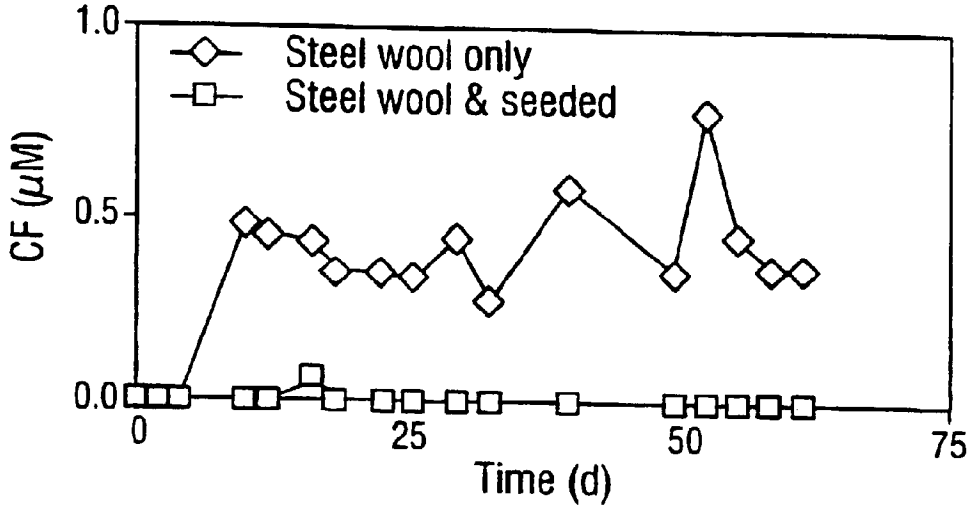


FIG. 14A

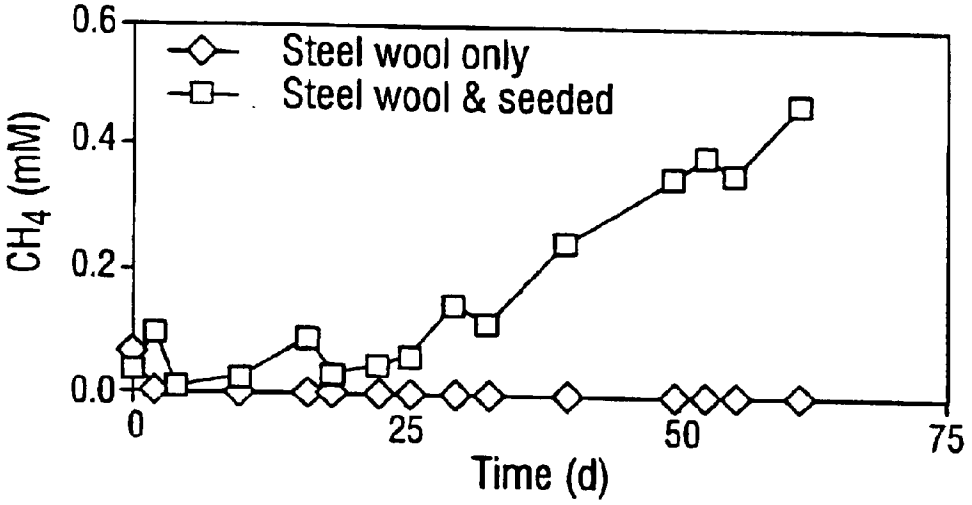


FIG. 14B

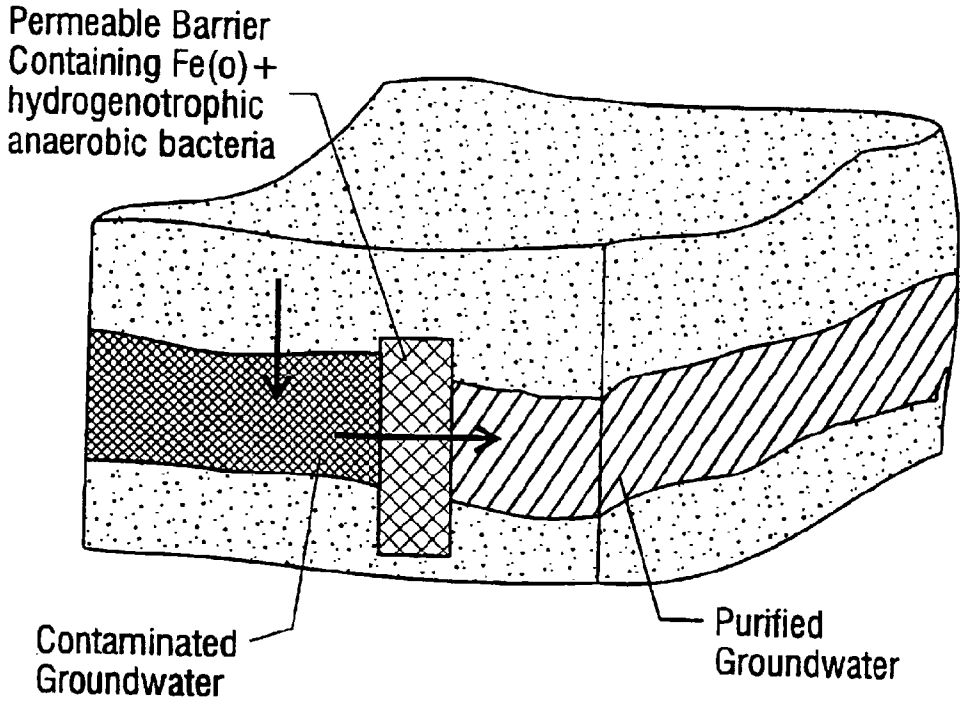


FIG. 15A

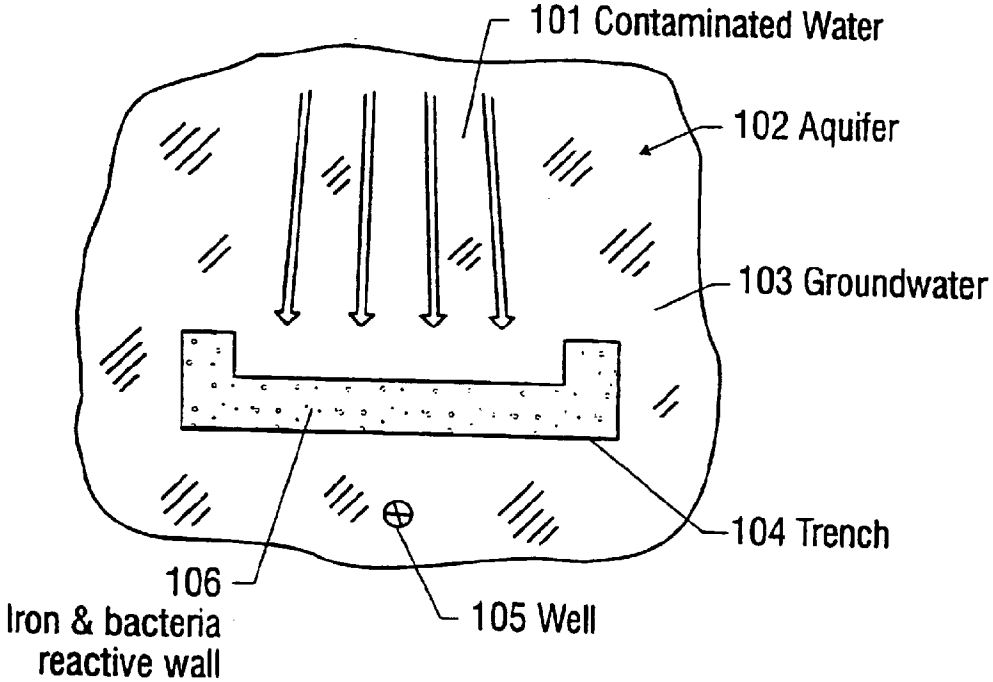


FIG. 15B

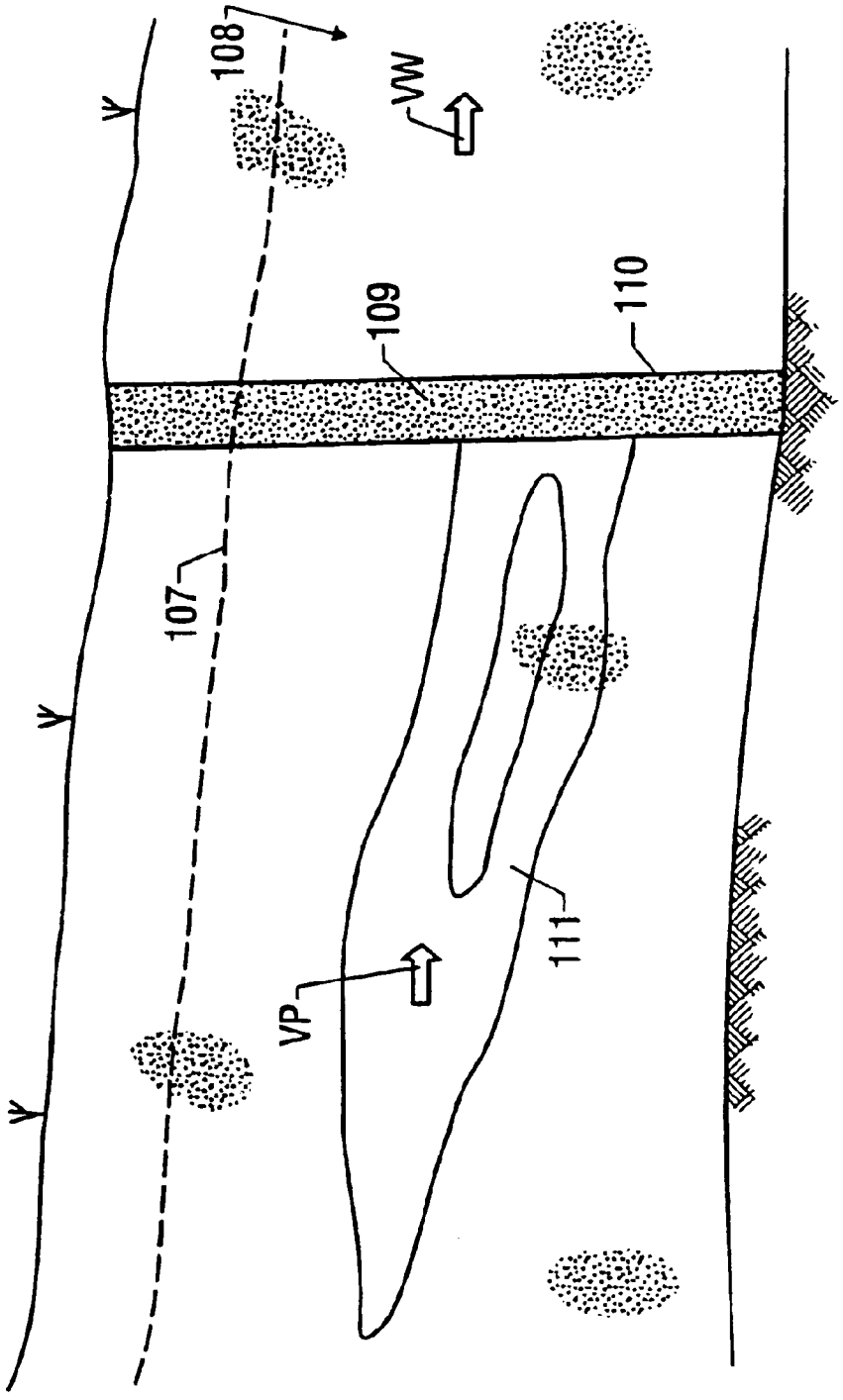


FIG. 15C

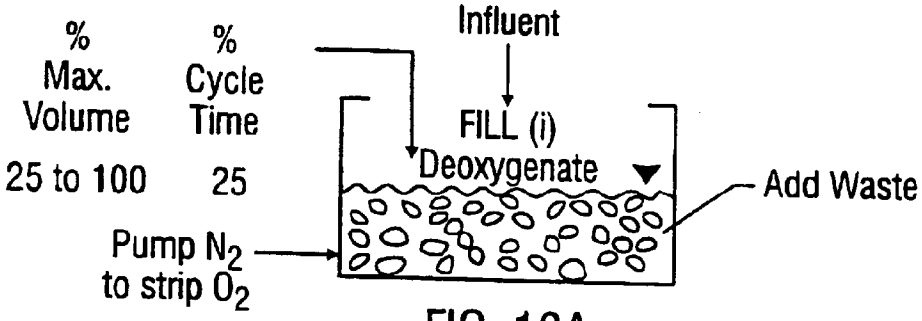


FIG. 16A

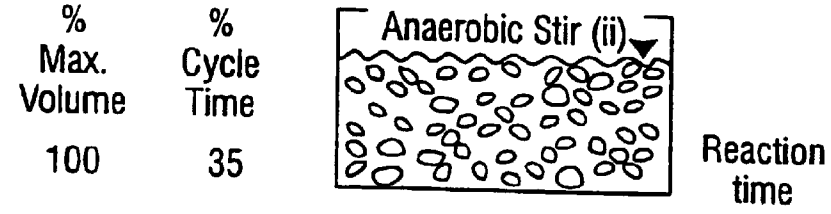


FIG. 16B

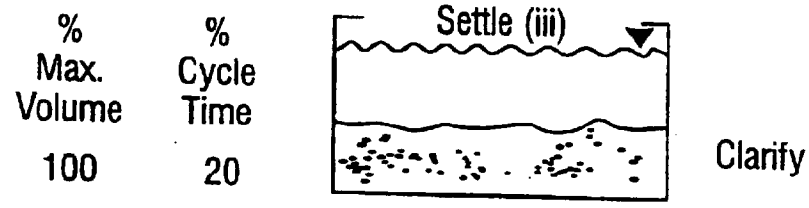


FIG. 16C

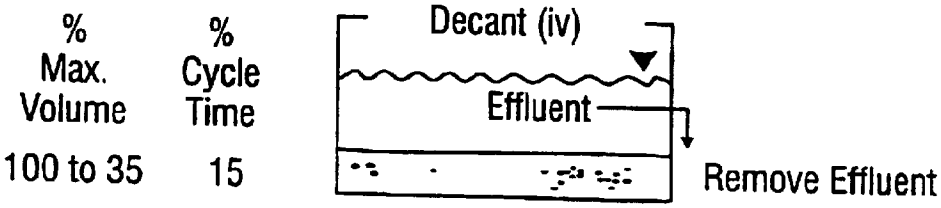


FIG. 16D

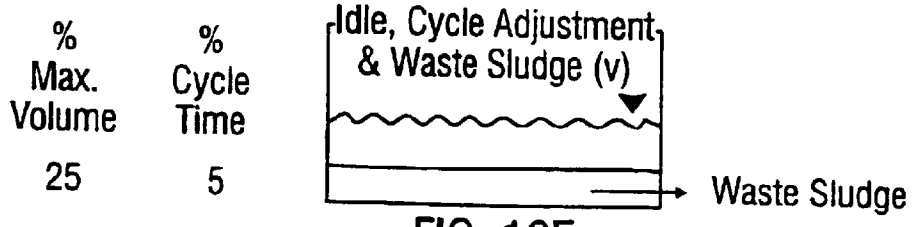


FIG. 16E

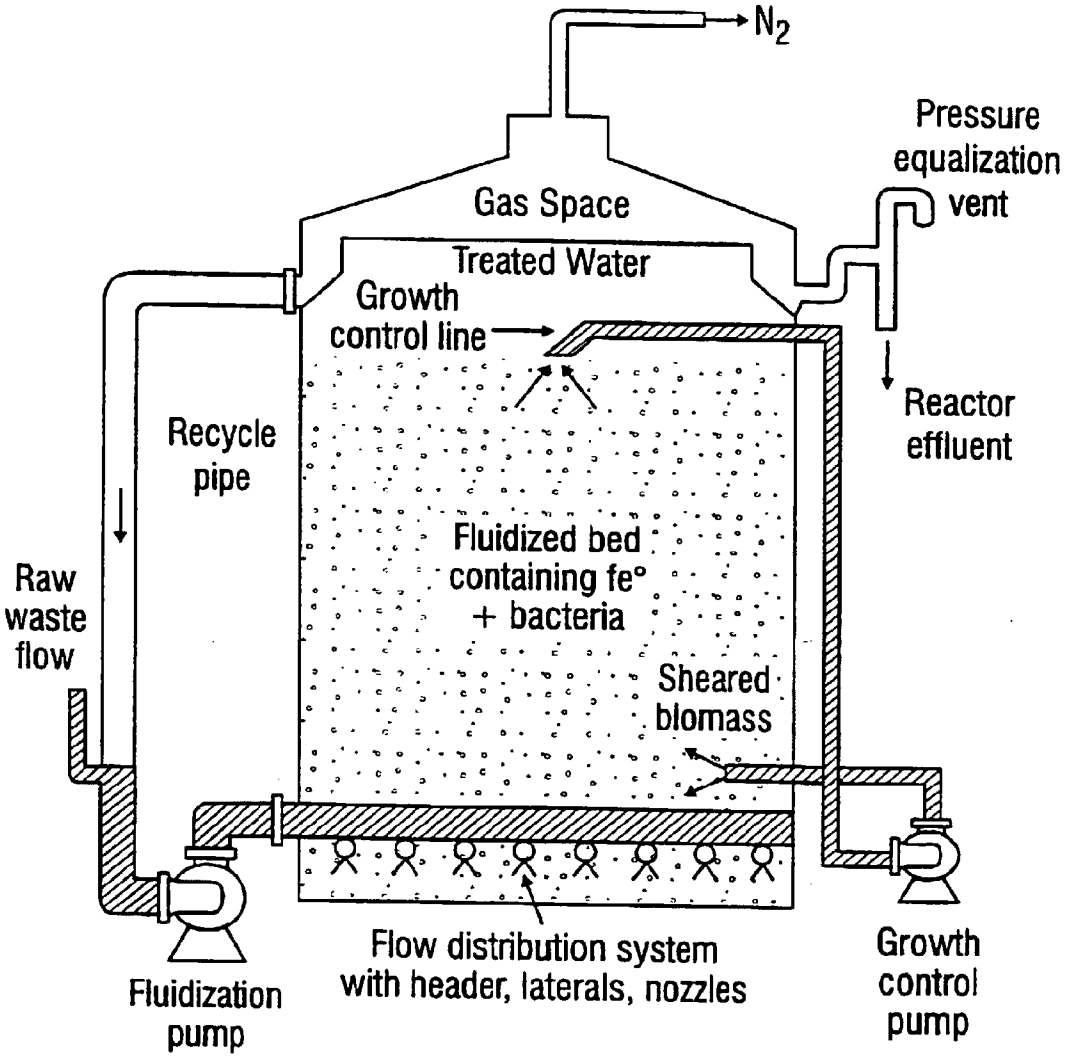

N_2
Pressure equalization vent
Gas Space
Treated Water
Growth control line
Recycle pipe
Reactor effluent
Fluidized bed containing fe° + bacteria
Raw waste flow
Sheared biomass
Flow distribution system with header, laterals, nozzles
Fluidization pump
Growth control pump

FIG. 17

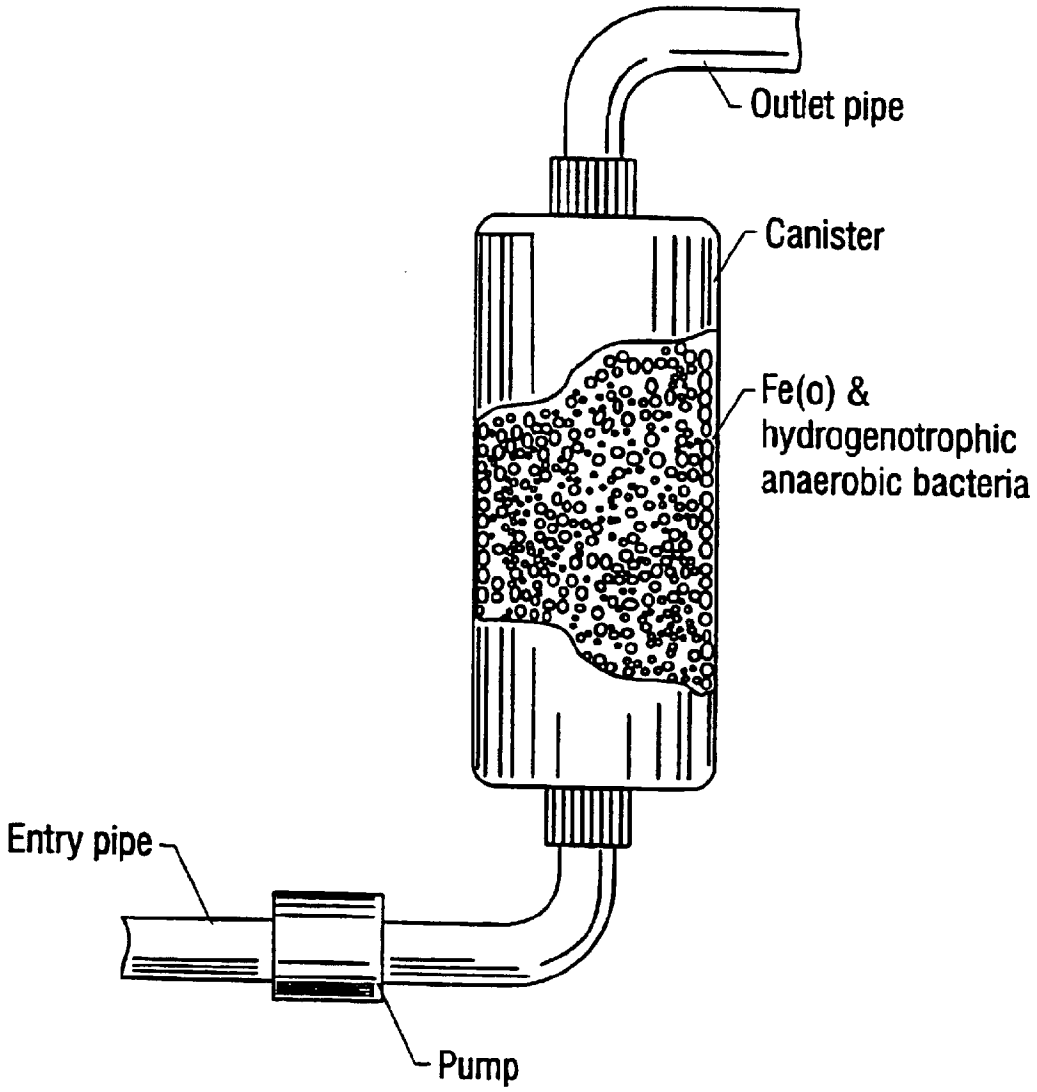

Outlet pipe
Canister
Fe(o) &
hydrogenotrophic
anaerobic bacteria
Entry pipe
Pump

FIG. 18

FE(O)-BASED BIOREMEDIATION OF AQUIFERS CONTAMINATED WITH MIXED WASTES

This is a 371 application of co-pending application Serial No. PCT/US98/08196, filed Apr. 24, 1998, which claims priority to U.S. provisional application No. 60/044,810, filed Apr. 25, 1997.

The present invention is a continuing application of U.S. Provisional Patent Application Serial No. 60/044,810 filed Apr. 25, 1997, the entire contents of which is specifically incorporated by reference in its entirety.

The United States government has rights in the present invention pursuant to Contract No. R-819653-01 from the Environmental Protection Agency.

BACKGROUND OF THE INVENTION

1.1 Field of the Invention

The present invention relates generally to the field of bioremediation. More particularly, it concerns compositions, methods and apparatus for the removal of nitrogenous and halocarbon pollutants from environmental sources including agricultural areas, soils, ground and surface water, sewage, sludges, landfill leachates, and wastewater. In particular embodiments, the invention discloses and claims compositions comprising zero-valent iron and hydrogenotrophic bacteria for use in removing target contaminants by a synergistic combination of abiotic and biological reductive mechanisms.

1.2 Description of Related Art

1.2.1 Abiotic Processes

Various abiotic processes have developed in recent years for the remediation of hazardous environmental pollutants (National Research Council, 1994). One process for abiotic remediation of organic and inorganic pollutants has been developed using zero-valent iron-[Fe(0)] mediated processes.

In this process, as elemental iron is oxidized (corrodes), electrons are released according to the following equations:

$$Fe^0 \rightarrow Fe^{2+} + 2e \quad \text{(Equation 1)}$$

These electrons are available for a variety of reduction-oxidation (redox) reactions. Water is reduced to produce hydrogen gas and alkalinity in the form of OH^-, with the net reaction resulting in a pH increase:

$$2H_2O + 2e^- \rightarrow H_2 + 2OH^- \quad \text{(Equation 2)}$$

$$Fe^0 + 2H_2O \rightarrow Fe^{2+} + H_2 + 2OH^- \quad \text{(Equation 3)}$$

Encouraging results in both laboratory and field experiments have stimulated a rapid increase in the use of Fe(0) as a reactive material to treat reducible contaminants (so-called reactive Fe(0) barriers). This approach has been used to degrade waste chlorinated solvents (e.g., Gillham and O'Hannesin, 1994; Johnson et al., 1996; Sweeny, 1980) and nitrate (Till et al., 1998). Reducible heavy metals such as Cr(VI) can also be removed from aqueous solution by reduction to less toxic forms (e.g., Cr(III)) and subsequent precipitation and immobilization, using Fe(0) as the sacrificial metal (Gould, 1982; Khudenko, 1987; Powell et al., 1995; Rickard and Fuerstran, 1968).

Semipermeable reactive Fe(0) barriers have been attractive for groundwater remediation in that they conserve energy and water, and through long-term low operating and maintenance costs, are considerably less costly than conventional cleanup methods. Fe(0) can be placed in the path of a contaminant plume, either on a trench (O'Hannesin and Gillham, 1992), buried as a broad continuous curtain (Blowes et al., 1995), or injected as colloids (Kaplan et al., 1994), to name a few options. However, the efficacy of Fe(0) systems can be limited by (site-specific) slow rates of reaction and by the potential accumulation of products of equal or greater toxicity (Matheson and Tratnyek, 1994; NRC, 1994; Roberts et al., 1996).

1.2.1.1 Chromium Remediation Using Abiotic Processes

Depending upon solution chemistry and pH, Cr(VI) can be present in the form of CrO_4^{2-}, $HCrO_4^-$, H_2CrO_4, and $Cr_2O_7^{2-}$. All of this hexavalent chromium species could be reduced to the less toxic, less mobile trivalent form, which is removed from solution as the hydroxide (i.e., $Cr(OH)_3$) under most conditions, using Fe(0) (Gould, 1982; Khudenko, 1987; Powell et al., 1995; Rickard and Fuerstran, 1968):

$$2Cr^{6+} + 6e^- \rightarrow 2Cr^{3+} \quad \text{(Equation 4)}$$

$$3Fe^0 \rightarrow 3Fe^{2+} + 6e^- \quad \text{(Equation 5)}$$

$$2Cr^{3+} + 6OH^- \rightarrow 2Cr(OH)_3 \quad \text{(Equation 6)}$$

$$2Cr^{6+} + 3Fe^0 + 6OH^- \rightarrow 2Cr(OH)_3 + 3Fe^{2+} \quad \text{(Equation 7)}$$

The increase in pH caused by iron corrosion (Equation 3) is thus beneficial in removing Cr(III) from solution.

When present in the environment, it is possible for the various species of Cr(VI), and Cr(III) to be sorbed to soils and sediments. It has been shown that Cr(VI) can be reduced to Cr(III) spontaneously by soil organic matter and/or by microorganisms under reducing conditions (Wang et al., 1989; Ishibashi et al., 1990; Yamamoto et al., 1993). Similarly, it has been shown that U(VI) can be reduced to U(IV) by microorganisms (Lovely and Phillips, 1992a and 1992b; Gorby and Lovely, 1992; Thomas and Macaskie, 1996). Once Cr(VI) is reduced to Cr(III) whether by soil organic matter or zero-valent iron, it is highly unlikely (due to kinetic constraints) for it to be oxidized once again. Only in the presence of freshly precipitated manganese oxides (MnO_2) or a strong oxidant like Fenton's reagent (hydroxyl radicals) can Cr(III) be reoxidized.

Fe(0) has shown significant promise in reducing, and thus removing from solution, Cr(VI) (e.g., Blowes et al., 1995; Powell et al., 1995; Gould, 1982). Presently, however, only one field site (Elizabeth City, N.C., Coast Guard site) exists where a reactive Fe(0) barrier is being evaluated to contain and remediate a groundwater plume contaminated with both Cr(VI) and TCE. (Morrison and Spangler, Roy E. West Geotech, Grand Junction, Colo.).

1.2.1.2 Uranium Removal Using Abiotic Processes

Uranium generally exists as the uranyl cation (UO_2^{2+}) in soils and groundwaters. It is tightly bound to soil and aquifer media at pH values greater than 6.0. However, it can be complexed by sulfate and organic ligands as well. Longmire et al. (1990) found that the predominant species in acidic uranium mill tailings deposits of New Mexico and Colorado was uranyl disulfate, $UO_2(SO_4)_2^{2-}$; uranyl sulfate aqueous complex, $UO_2(SO_4)^0$, uranyl divalent cations, and uranyl biphosphate, $UO_2(HPO_4)_2^{2-}$; in that order. Once U(VI) is reduced to U(IV), it becomes much less mobile, similar to

chromium. Immobilization is caused by the precipitation of uranium dioxide, and by strong sorption of U(IV) species to soils and sediments. Similar to Cr(III), once uranium has been reduced, it is not likely to become mobilized again unless the pH is reduced or a strong oxidizing agent is encountered. Treatment with zero-valent iron removes dissolved oxygen and increases the pH; both conditions which aid the chemical reduction of chromium and uranium and which keeps them immobilized.

Hexavalent uranium can also be reduced to the less mobile U(IV) form which is removed from solution as the oxide under most conditions:

$$U^{6+}+2e^{-}\rightarrow U^{4+} \quad \text{(Equation 8)}$$

$$U^{4+}+4OH^{-}\rightarrow UO_2+2H_2O \quad \text{(Equation 9)}$$

Here again, the increase in pH caused by Fe(0) corrosion (Equation 3) is conducive to U(IV) precipitation from solution (Lovely and Phillips, 1992a; Morrison et al., 1995; Thomas and Macaskie, 1996).

Unfortunately, no reports in the literature describe the use of reactive Fe(0) barriers to reduce and remove U(VI), although a pilot facility in Durango, Colo., is presently being tested (S. Morrison of Roy E. West Geotech, Grand Junction, Colo.).

1.2.1.3 Removal of Polychlorinated Organics Using Abiotic Processes

Polychlorinated organics can also be reduced using the e^{-} generated by iron corrosion by replacing Cl atoms with hydrogen atoms. This form of reductive dechlorination is termed hydrogenolysis (Vogel et al., 1987). Using carbon tetrachloride (CT) as an example, first chloroform (CF) and then dichloromethane (DCM) are formed:

$$CCl_4+2e^{-}+H^{+}\rightarrow CHCl_3+Cl^{-} \quad \text{(Equation 10)}$$

$$CHCl_3+2e^{-}+H^{+}\rightarrow CH_2Cl_2+Cl^{-} \quad \text{(Equation 11)}$$

Several researches have shown that DCM is a "dead-end" product of abiotic treatment of CT or CF with Fe(0) (Helland et al., 1995; Matheson and Tratnyek, 1994). Thus, while abiotic processes are able to reduce some organic compounds, the process often results in endproducts which are toxic, themselves. As such, there are limitations to the use of abiotic processes alone in the remediation of organic compounds from the environment.

TCE is reportedly converted to ethene by hydrogenolysis using Fe(0), generally without the build-up of intermediates (Orth and Gillham, 1996), although trace amounts of chlorinated acetylenes could be formed by TCE dihaloelimination (Roberts et al., 1996). A simplified stoichiometry for TCE hydrogenolysis would be:

$$CHCl{=}CCl_2+6e^{-}+3H^{+}\rightarrow CH_2{=}CH_2+3Cl \quad \text{(Equation 12)}$$

$$3Fe^{0}\rightarrow 3Fe^{2+}+6e^{-} \quad \text{(Equation 13)}$$

$$CHCl{=}CCl_2+3Fe^{0}+3H^{+}\rightarrow CH_2{=}CH_2+3Fe^{2+}+3Cl^{-} \quad \text{(Equation 14)}$$

Unfortunately, the fact that each of the above reactions is thermodynamically feasible does not guarantee that the reactions are feasible for the removal of such compounds from solution using only abiotic processes. The contaminants must be sufficiently reactive that suitable transformation take place during the time the contaminated groundwater flows through the treatment zone. Moreover, observed removal rates may reflect a number of processes other than chemical reaction at the Fe(0) surface, including mass transport to the surface, adsorption of reactants, and desorption and mass transport of products from the surface (Helland et al., 1995). A positive correlation has been suggested between the mixing rate in batch reactors and reductive dechlorination rate (Matteson and Tratnyek, 1984), presumably due to faster mass transport resulting from the decreased thickness of the diffusion layer. That removal rates might be mass transfer rather than reaction limited, suggests the importance of enhancing contact between Fe(0) and the target contaminant.

1.2.2 Biotic Processes

1.2.2.1 Biotic Removal of Chromium and Uranium

Biotic reduction of Cr(VI) and U(VI) may be indirect or direct. Microbes can be indirectly responsible for metal reduction by producing relatively strong reductants like H_2S from SO_4^{2-} and Fe(II) from Fe(III). In most cases, abiotic reduction using H_2S and Fe(II) is much slower than direct biotic reduction (Kriegman-King and Reinhard, 1994; Doong and Wu, 1992). A number of studies have shown that several microorganisms can directly (intracellularly) reduce Cr(VI) to Cr(III) using Cr(VI) as an electron acceptor during microbial respiration (Wang et al., 1989; Ishibashi et al., 1990; Yamamoto et al., 1993). Similarly, it has been shown that U(VI) can be reduced to U(IV) by microorganisms (Lovely and Phillips, 1992a and 1992b; Gorby and Lovely, 1992; Thomas and Macaskie, 1996). For the most part, metal reductases have been implicated in these studies. However, there is evidence that such metals can be used as electron acceptors for growth (Ormerod, 1991). These organisms, GS-15 and *Shewanella putrefaciens,* can also use nitrate and Fe(III) as terminal electron acceptors.

1.2.2.2 Biotic Processes for Remediating Halocarbons

It has also been shown that a variety of microbes can catalyze the reduction of many chlorinated hydrocarbons. A variety of chlorinated aliphatic hydrocarbons are biotransformed by pure and mixed methanogenic (Bagley and Gossett, 1996; Bouwer et al., 1981; Bouwer and McCarty, 1983; Gossett, 1985; Egli et al., 1987; Hughes and Parkin, 1996; Krone et al., 1989a;b; Mikesell and Boyd, 1990) and non-methanogenic, anaerobic cultures (Egli et al., 1987; Egli et al., 1988; Galli and McCarty, 1989; Egli et al., 1990; Fathepure and Tiedje, 1994). Unfortunately, however, with most biological reactions, when reductive dechlorination is the dominant pathway, intermediates will accumulate (e.g., chloroform and dichloromethane from CT biotransformation; vinyl chloride and the dichloroethenes from TCE and PCE biotransformation). These metabolites are often of more concern than the parent compounds, and thus, the art remains limited with respect to biological treatment of chlorinated hydrocarbons. It should be noted, however, that tetrachloroethene (PCE) and TCE can be used as electron acceptors for growth of some anaerobic organisms with the end products being ethene or ethane (Holliger et al., 1993; Holliger, 1995; Scholz-Muramatsu et al., 1995).

Anaerobic conditions are required to produce the H_2 from F(0) corrosion and support the growth of useful anaerobic bacteria. Dissolved oxygen, which may be present is some aquifers, is toxic to anaerobes and may inhibit their activity. Nevertheless, oxygen is quickly depleted by aerobic corrosion of Fe(0) as shown by Helland et al. (1995):

$$2Fe^{0}+O_2+4H^{+}\rightarrow 2Fe^{2+}+2H_2O \quad \text{(Equation 15)}$$

This reaction induces anoxic conditions that are favorable for anaerobic biotransformations.

1.2.2.3 Chlorinated Solvents

Chlorinated solvents such as trichloroethylene (TCE), heavy metals such as hexavalent chromium, and radionuclides such as hexavalent uranium, are among the most common contaminants found at DOE sites (Riley et al., 1992). Mixtures of such contaminants have been found in soils and sediments at 11 DOE facilities and in the groundwater at 29 sites. While numerous physical-chemical and biological processes have been proposed to manage DOE contaminated sites, many of these approaches are only marginally cost-effective and/or have detrimental side effects on environmental quality, particularly pump-and-treat processes (National Academy of Science, 1992). Consequently, there is a need to develop improved alternatives for the remediation of sites containing these contaminants.

One alternative was the use of elemental (or zero-valent) iron (Fe(0)) in the development of strictly abiotic processes. Although the reactivity of Fe(0) with chlorinated compounds was recognized as early as 1925 (Rhodes and Carty, 1925), only recently has this process received considerable attention for treating waste chlorinated solvents (e.g., Gillham and O'Hannesin, 1994; Johnson et al., 1996; Sweeny et al., 1980). Reducible heavy metal ions (e.g., hexavalent chromium) and nucleotides (e.g., hexavalent uranium) can also be removed from aqueous solution by reduction and subsequent precipitation using Fe(0) as the sacrificial metal (i.e. "cementation") (e.g., Gould, 1982; Khudenko, 1987; Rickard and Fuerstran, 1968). Results from laboratory and pilot studies awakened considerable national and international interest in the use of Fe(0) as a reactive material (so-called reactive Fe(0) barriers) to minimize subsurface migration of such reducible contaminants. Passive, semipermeable reactive walls are also particularly attractive in that they conserve energy and water, and through long-term low operating and maintenance costs, are considerably less costly than conventional cleanup methods.

Nevertheless, knowledge on the applicability and limitations of reactive Fe(0) barriers is limited, and the feasibility of this process to treat mixtures of chlorinated solvents, heavy metals, and radionuclides has not been demonstrated in the art. One limitation of abiotic reduction with Fe(0) alone to remove some polychlorinated compounds such as carbon tetrachloride is the accumulation of transformation products of equal or perhaps greater toxicity (Helland et al., 1995; Matheson and Tratnyek, 1994; Roberts et al., 1996).

Several reports have suggested that a wide variety of microbes can facilitate reductive dechlorination of polychlorinated organics (see e.g., Bouwer et al., 1981; Krone et al., 1989a;b; Holliger et al., 1993; Vogel et al., 1987). Some microbes have also been shown to facilitate the reduction and immobilization of reducible heavy metal ions, e.g., Cr(VI) (Wang et al., 1989) and radionuclides e.g., U(VI) (Lovely and Phillips, 1992a; 1992b). Anaerobic microorganisms have also been shown to respire nitrate and sulfate originating from waste acids at uranium mill tailings (e.g., Durango, Colo., and Tuba City, Ariz.), which is a major challenge facing the Uranium Mill Tailing Remediation Act (UMTRA) program. However, the availability of appropriate primary substrates has limited the success of these biotic transformations in situ. In particular, many of these contaminants are toxic to a variety of bacterial strains when present at high concentrations, and the rate of remediation by these organisms has been disappointing.

1.3 Deficiencies in the Prior Art

There are several chemical and biological technologies that remove nitrates, and other inorganic compounds as well as organic compounds from water and wastewaters. However, these processes are marginally cost-effective and/or have detrimental side-effects on water quality. For example, physical-chemical processes involving membrane filtration technologies or ion exchange resins are often prohibitively expensive and merely transfer the inorganics, such as nitrates, from one phase to another, thus creating a disposal problem, and creating large quantities of brines.

While biological denitrification processes can convert nitrate to innocuous dinitrogen gas and are typically less expensive, they have adverse side-effects on water quality due to residual organic compounds used to support heterotrophic biological activity and excessive biomass production potentially contaminating the treated water. Therefore, what is lacking in the prior art are effective means for the bioremediation of aqueous environments, particularly with respect to denitrification and the removal of organic compounds such as halocarbons, using systems which do not adversely affect the water quality.

Unfortunately, the efficacy of strictly abiotic processes relying on Fe(0) alone is limited by (site-specific) slow rates of reaction and by the accumulation of products of equal or greater toxicity than the pollutants to be remediated (Matheson and Tratnyek, 1994; NRC, 1994; Roberts et al., 1996). Early work by scientists suggested the coupling of anaerobic oxidation of Fe(0) to a reduction of chloroform was possible using methanogenic bacteria, but no evidence suggested the use of such synergistic processes for inorganic compounds, or organic compounds other than chloroform (Weathers et al., 1995a;b)

2.0 Summary of the Invention

The present invention overcomes these and other limitations in the prior art by combining Fe(0) technologies with hydrogenotrophic microorganisms to exploit favorable biogeochemical interactions to detoxifying a variety of inorganic and organic compounds. The inventors' suprising finding that such biogeochemical interactions could facilitate the reduction of not only inorganic and metal-ion containing compounds, but also haloaromatic, nitroaromatic, and organic pesticides has facilitated a revolutionary advance in the area of microbial-based bioremediation methods. Disclosed and claimed herein are devices and methods for the bioremediation of environmental sites and aqueous solutions using a synergistic combination of biotic and abiotic processes. In particular, the invention concerns iron-supported autotrophic methods which utilize a device comprising a composition containing zero-valent iron and a culture of one or more species of hydrogenotrophic bacteria to remove target contaminants.

2.1 Hydrogenotrophic Bacterial/Zero-Valent Iron Devices

In a first embodiment, the invention concerns a device which comprises a composition of zero-valent iron and a culture of one or more hydrogenotrophic bacteria. The hydrogenotrophic bacteria preferably comprise one or more species of bacteria selected from the group consisting of Acetobacterium spp., Achromobacter spp., Aeromonas spp., Acinetobacter spp., Aureobacterium spp., Bacillus spp., Comamonas spp., Dehalobacter spp., Dehalospirillum spp., Dehalococcoide spp., Desulfurosarcina spp., Desulfomonile

spp., Desulfobacterium spp., Enterobacter spp., Hydrogenobacter spp., Methanosarcina spp., Pseudomonas spp., Shewanella spp., Methanosarcina spp., Micrococcus spp., and Paracoccus spp. Alternatively, hydrogenotrophic bacteria present in anaerobic sludge or ariaerobic sediments may also be used in the practice of the invention.

Exemplary hydrogenotrophic bacteria include one or more strains of bacteria selected from the group consisting of *Acetobacterium woodi, Aeromonas hydrophila, Aeromonas sobria, Alcaligenes eutrophus, Comamonas acidovorans, Dehalococcoide resirictus, Dehalococcoide multivorans, Dehalococcoide ethenogene, Desulfobacterium tiedje, Enterobacter agglomerans, Hydrogenobacter thermophilus, Methanosarcina barkeri, Methanosarcina mazei, Methanosarcina thermophila, Paracoccus denitrificans, Pseudomonas aureofaciens, Pseudomonas maltophilia, Pseudomonas mendocina,* and *Shewanella putrefaciens.*

In illustrative embodiments, the inventors have shown that hydrogenotrophic bacterial stains such as *Paracoccus denitrificans* ATCC17741, *Paracoccus denitrificans* ATCC35512, *Paracoccus denitrificans* ATCC13543, or *Paracoccus denitrificans* ATCC19367 are particularly useful in formulating the compositions of the invention. In certain embodiments, the inventors contemplate the use of a mixed culture of one or more of the disclosed microorganisms. Such mixed cultures may comprise two or more hydrogenotrophic organisms, and may also include one or more strains, species, or genera of non-hydrogenotrophic bacteria.

The zero-valent iron of the composition is preferably iron, iron alloy, or iron bimetal. in It may be in the form of metal filings, shavings, turnings, powder, mesh, steel wool, beads, rods, or pellets (exemplary sources include e.g, Malinkrodt, Fisher Scientific, Master Builder, Aldrich Chemical Co., scrap metal suppliers, and the like). Iron bimetallics, composed of a combination of Fe(0) and any of the following: Ni(0), Zn(0), Pt(0), and Pd(0), are also desirable for the compositions and processes of the invention, as are iron-based metallic alloys. The metal may be immobilized in a gel, matrix, or other medium, may be in combination with one or more zeolites or minerals, and may be embedded, or immobilized onto glass, ceramic, cloth, plastic, fiber, non-metal, metalloids, crystals, polymers, and the like. In fact, any formulation of the zero-valent iron which permits contact with or proximity to the hydrogenotrophic bacterial culture, and which permits or facilitates the oxidation of the metal and the liberation of hydrogen gas to be uptaken and utilized by the bacterial culture is contemplated by the inventors to be useful in formulating the particular iron-culture composition.

In preferred embodiments the culture of hydrogenotrophic bacteria (either alone or in combination with other microorganisms) is immobilized, mixed with, or in close proximity to the solid or semi-solid substrate(s) that comprise the Fe(0) compound. The solid substrate may be, but is not limited to, matrices, columns, chromatographic media, glass or plastic beads, plates or surfaces, acrylic beads, acrylamides, polyacrylamides, beaded agarose, Sepharose, tubing, vials, metal supports, mesh, fibers, polymers, ceramics, or cloth and the like. The Fe(0) component may be attached to the solid substrate by any suitable means known to those of skill in the art. Alternatively, the iron itself, or the device housing may serve as a substrate for the growth of the bacterial cells. The device may be a cartridge, a filter, a vessel (including e.g., a reactor, flask, beaker, funnel, bottle, cannister, or tank), a flow-through tubing, radiator, fermenter, or such like. In the case of in situ remediation, the device may be comprised within a system or remediation apparatus, and may comprise a reactive barrier, a membrane, a cylindrical barrier, a gate-and-funnel apparatus, or any other apparatus suitable for placement in an environmental site, and suitable for providing a means for containing the bacterial-Fe(0) composition within the device.

The Fe(0)-containing compound may typically be present at a concentration of from between about 1% and about 99%, more preferably from between about 10% and about 80%, or more preferably from between about 15% and about 50% or so by weight. The iron compound may be alone, or may be added to a porous medium or semi-porous substrate. This medium may contain one or more aluminosilicate minerals (e.g., bentonite, montmorillonite, kaolinite, gibbsite, microline feldspar, albite feldspar) to enhance proton generation at the Fe(0) surface and accelerate corrosion, as described by Powell et al. (1997). The porous medium may also be amended with one or more zeolite minerals to retard the movement of halogenated and nitrated organic contaminants through reactive barriers. This increases both retention time and the removal efficiency. The medium may be formulated to promote adherence by the bacterial culture to the iron substrate, and may be formulated to promote growth or survival of the bacterial culture. Optionally, the medium may be formulated to contain one or more antifungal, antiviral, or antiparasitic agents to retard or prevent the growth of fungi, virus, or parasites in the composition. Also optionally, the composition may be augmented to provide one or more nutrients, vitamins, minerals, or substrates for utilization by the microbial colony. In certain instances, sorbants, such as charcoal, zeolites, or the like, may be added to the composition.

2.2 Methods for Bioremediation

In a second embodiment, the invention provides methods for detoxifying, decontaminating, altering, removing or reducing the concentration of one or more organic or inorganic compounds from an aqueous solution, leachate, runoff, aquifer, groundwater, surface water, well water, an environmental site, soils, and/or agricultural or industrial sources. In particular, compositions and methods are provided for removing, detoxifying, or reducing the concentration of one or more inorganic or organic compound (including nitrates, nitrites, sulfates, sulfites, strontium-, cesium-, chromium- and uranium-containing compounds, halocarbons, haloaromatics, nitroaromatics, and compounds containing one or more nitro- or nitroso-groups) from one or more such site. The methods encompass both in situ and ex situ remediation procedures, and provide both apparatus for large-scale remediation, and devices for remediation of particular pollutants. The inorganic and organic compounds may be naturally occuring pollutants, or may be introduced into the site by the hand of man. The compounds may be present in one or more sites, and may be present in such sites either in pure or nearly-pure form, or may be present along with a plurality of toxic or polluting compounds. Examples of such pollutants may include herbicides, pesticides, industrial chemicals, chemical manufacturing byproducts, byproducts of natural decomposition processes, human or non human wastes, landfill components, mining wastes or runoff, agricultural leachates, industrial runoff, fertilizers and fertilizer byproducts, poisons, and the like. Such pollutants may be present in an enviromental site as the result of a chemical spill, industrial or agricultural accident, pipeline and storage tank failures, derailment of train cars,

accidents involving motorized transport of chemicals, explosions, storage tank ruptures, acts of sabotage, and the like.

In an overall and general sense, the methods of the invention generally involve identifying or selecting an aqueous solution or a soil sample or an environmental site that is known to contain, or suspected of containing, or shown to contain, or shown to be susceptible to pollution by, one or more of the inorganic or organic contaminants as disclosed herein, and contacting the solution, soil, or site with a composition comprising a culture of one or more hydrogenotrophic bacteria and a zero-valent iron composition. The aqueous solution may be present in a pipeline, a water or sewage treatment facility, an aqueduct, drainage pond, settling basin, reservoir, storage vessel, or other man-made facility or the like. Alternatively, the aqueous solution may be an environmental site such as a lake, creek, river, stream, aquifer, pond, drainage ditch, or part of an agricultural area, irrigation system, industrial waste facility, landfill, or the like. The environmental site may include any of these areas, and may also include soils, subsurface areas, aquifer recharge zones, leachate areas, embankments in proximity to agricultural sites, water sources, groundwaters, wells, and the like. The environmental site may be defined as, or may be in proximity to, an industrial plant, a treatment plant, a mine, or mining facility, a processing plant, a construction site, a ranch, farm, or cultivated region, a potable or non-potable water supply, a pipeline or utility supply region, sewage facilities, drains, or pipelines, culverts, basins, storm drains, or flood plain control systems. In fact, the inventors contemplate that any site or aqueous solution that is suspected of containing, or is susceptible to pollution by, or is in proximity to a region either contaminated with, or polluted by, one or more inorganic and organic compounds may be a site chosen for remediation using the disclosed methods and compositions.

In certain embodiments, it will be preferable to provide devices which comprise the disclosed compositions to a source to be remediated. This is particularly preferred with the treatment methods are in situ, such as reactive barriers, and the like which are placed into a particular polluted site. Alternatively, where ex situ treatment is desired (e.g., in the treatment of water supplies, pipelines, drainage or collection facilities, storage tanks, sewage treatment plants, etc.) the inventors contemplate the use of the compositions in the manufacture of apparatus and water treatment devices to detoxify such systems. This is particularly desirable in the manufacture of water treatment cartridges, treatment facility machinery, and other apparatus which may be utilized to treat a contaminated sample not present in its native environment.

In one embodiment, the invention provides a method for removing or detoxifying nitroaromatic compounds from an aqueous solution. The method generally involves contacting an aqueous solution suspected of containing a nitroaromatic compound (such as trinitrotoluene, RDX, HMX, 2-aminodintrotoluene, 4-aminodinitrotoluene, and parathion) with a composition consisting of a culture of one or more hydrogenotrophic bacteria and an Fe(0) composition.

In a further embodiment, the invention provides a method for removing or dehalogenating a halocarbon in an aqueous solution. This method generally involves contacting an aqueous solution suspected of containing a halocarbon, such as chlorinated benzenes, trichloroethylene, perchloroethylene, dichloroethylene, vinyl chloride, chloroethane, bromoform, dichlorodifluoromethane, trihalomethanes, tetrachlorodibenzodioxin pentachlorophenol, chlorobenzoates, atrazine, 1,1,1-TCA, CCl_4, $CHCl_3$, DCM, or a PCB, with a composition comprising one or more autotrophic bacteria and an Fe(0) composition.

A further aspect of the invention is a method for removing, detoxifying, or reducing the concentration of a poison, herbicide fungicide, nematocide, or pesticide in an aqueous solution or an environmental site. This method generally involves contacting the solution or site suspected of containing such a chemical with a composition comprising one or more hydrogenotrophic bacteria and Fe(0). Exemplary pesticides remediable by these methods include methoxyclor, alachlor, metolachlor, lindane, DDT, DDE, DDD, dieldrin, aldrin, heptachlor, chlordane, 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid and atrazine.

In another embodiment, the invention provides a method for detoxifying, removing or reducing the concentration of redox-sensitive metal ions, such as strontium (II), cesium (I), chromium (VI), uranium (VI), technetium ( ), silver (I), or mercury (II) ions in an aqueous solution or an environmental site. This method generally involves contacting the solution or site suspected of containing one or more of such ions, either free in solution or complexed by organic ligands, with a composition comprising one or more hydrogenotrophic bacteria and Fe(0).

In a further embodiment, the invention provides a method for removing, detoxifying, or reducing the concentration of an inorganic compound such as nitrite, nitrate, sulfite, or sulfate in an aqueous solution or an environemental site. This method comprises contacting the solution or site suspected of containing such an inorganic compound with a composition comprising one or more hydrogenotrophic bacteria and Fe(0).

Another aspect of the invention is a method for reducing a nitroaromatic or a haloaromatic compound in a sample. The method involves selecting or identifying a sample that contains or is suspected of containing one or more such compounds with a composition comprising one or more hydrogenotrophic bacteria and Fe(0).

The present invention further provides methods for the bioaugmentation of in situ reactive permeable barriers, packed or fluidized bed reactors for industrial wastewater or landfill leachate treatment, sand filtration technologies for drinking water treatment, anaerobic digestion for sludge treatment, cylindrical reactive barriers surrounding groundwater wells for well head protection, and combinational approaches using membrane technologies well-known in the art for purification and treatment of water, and in particular, municipal, potable, or water suitable for animal and human consumption.

The efficiency of each method may be calculated as the ratio of the amount of contaminant removed divided by the amount of contaminant initially present in the sample. The rate of decomination may be estimated as the amount of contaminant removed divided by its retention time in the particular treatment zone utilized for remediation. The amount of contaminants present in a site and the amount remaining in a site following treatment may be measured using standard analytical techniques developed for each of the particular contaminants.

2.3 Apparatus and Devices for Synergistic Bioremediation

The inventors contemplate at least two different approaches for employing the present methods for the

bioremediation of water, agricultural extracts or soil leachates, surficial sediments, surface waters, aquifers, groundwaters, springs, and other environmental aqueous areas contaminated with pollutants which are remediable utilizing these methods.

In one embodiment, the invention provides in situ remediation methods, and in particular, the use of permeable and semipermeable barriers. In situ permeable barriers, such as those described in Great Britain Patents GB 2238533A and GB 2255081A represent illustrative embodiments adaptable to the methods of the present invention. The disclosed technique is substantially improved using the present method through the pumping of bacteria either into the groundwater up-gradient from the permeable barrier, or directly into the barrier (FIG. **15**A, FIG. **15**B, and FIG. **15**C). Preferred permeable barrier dimensions range from about 2 to about 6 ft thick, about 25 to about 50 ft long, and from about 15 to about 30 ft deep.

In another embodiment, the invention provides methods and apparatus comprising a treatment wall in a trench. In the simplest case, a trench of the appropriate width can be excavated to intercept the contaminated strata and backfilled with reactive material. The reactive material would consist of Fe(0) mixed with some of the alumniosilicate and/or zeolite minerals and with some anaerobic hydrogenotrophic bacteria mentioned previously (FIG. **2**).

Shoring of the trench and use of an appropriate slurry or steel sheet piling may be required for excavation to depths greater than 10 feet. Unlike conventional approaches for groundwater cut-off walls that utilize a soil-bentonite slurry, installation of permeable treatment walls may require the use of biodegradable polymers instead of bentonite or cement to avoid the problem of plugging the wall with residual slurry material (Vidic and Pohland, 1996).

To overcome potential limitations to the life expectancy of the added Fe(0), the reactive media can be placed in the subsurface in removable cassettes, as described by MSE (1996). A temporary sheet pile box or a larger diameter caisson can be installed into the subsurface and the screen panels can be placed on the up- and down-gradient sides, while impermeable panels are placed on the lateral sides. Steel rail guides for the cassettes are installed within this interior compartment and the temporary sheet piles or caisson are removed. The cassette can be a steel frame box (e.g., 8 ft long, 5 ft wide, 1.5 ft thick) with two opposing screened sides and two impermeable sides which is filled with the reactive media and lowered into the cavity. By allowing replacement of cassettes with depleted reactive media, the remediation system operation life can be extended near indefinitely.

In a further embodiment, the invention provides methods and apparatus using an injected treatment zone. A treatment zone which (unlike the treatment wall) is not confined within strict boundaries can be established by using injection wells or by hydraulic fracturing (FIG. **3**).

Well systems typically involve injection of fluids or fluid/particulate mixtures for distribution into a treatment zone within the target area of the aquifer (Vidic and Pohland, 1996). Potential advantages of this approach are that there is no need to construct a trench and possible aquifer access at greater depths.

In a further embodiment, the invention provides methods and apparatus using a "funnel and gate" system: In these embodiments, low-permeability cut-off walls (e.g., 10^{-6} cm/s) could be installed with gaps that contain in situ reactive zones (FIG. **4**). Cut-off walls (the funnel) modify flow patterns so that groundwater primarily flows through high conductivity gaps (the gates). The cut-off wall could be slurry walls, sheet piles, or solid admixtures applied by soil mixing or jet grouting. The gate would consist of a treatment wall similar to those described above.

Alternatively, in situ remediation may be achieved using barrier technologies such as that of and the compositions of the present invention may also be placed in the path of a contaminant plume, either on a trench (O'Hannesin and Gillham, 1992), buried as a broad continuous curtain (Blowes et al., 1995), or injected as colloids (Kaplan et al., 1994).

In general, these methods involved the use of a large plastic wall or retainer, which is inserted into the subsurface, for example, in a "funnel and gate" design. This method permits the installation and removal of one or more cartridges into the system, without the need for changing the entire system. In this scheme, the inventors contemplate utilizing one or more cartridges which each comprise an Fe(0) substrate and an autotrophic bacterial population. Then, if the bacterial culture or iron substrate needs amending, replenishing, or replacement, the cartridge may be retrieved and a new one re-inserted with fresh substrate and microorganisms. When indicated, natural zeolite materials may be used within the matrix of these reactive barriers to sorb target pollutants and allow for a longer retention time, thus allowing for thinner barriers.

The second general embodiment concerns ex situ treatment methods and devices. Ex situ treatment includes various bioreactor modes and schemes such as sequencing batch reactors (SBR) (FIG. **16**), fluidized beds (FIG. **17**), and flow-through packed columns (FIG. **18**).

The SBR is a periodically operated batch, fill-and-draw reactor containing a support structure for Fe(0) and the bacteria (FIG. **13**). Each reactor in an SBR system has five discrete cycles in each cycle. For a nitrate removal scheme these would include, (i) fill/deoxygenate, anaerobic stir and react, (iii) settle, (iv) decant, and (v) idle, cycle adjustment and waste sludge. An advantage of this type of treatment scheme is the flexibility in reaction times depending on the waste being treated. Fluidized bed (FIG. **14**) and flow-through packed columns (FIG. **15**) are attached growth reactors where contaminated water is continuously pumped through a reactor containing Fe(0) and bacteria. In a fluidized bed reactor, a fine-grained iron would serve as the support media for bacterial growth. Fluidization significantly increases the specific surface area and allows for high biomass concentrations in the reactor. It also reduces the clogging potential when the contaminated fluid contains suspended solids. Flow-through packed column reactors contain Fe(0) support structures to allow for biological growth and attachment.

In general, the inventors contemplate that any apparatus which comprises at least a first inlet port, at least a first outlet port and at least one compartment that comprises an Fe(0)-hydrogenotrophic bacterial composition may be developed to remediate target pollutants from an aqueous solution passed through the device. Exemplary devices include flow-through bioreactors and fluidized bed reactors. These devices may also include cartridges or self-contained modules which form a part of a larger apparatus designed for the treatment of a water source or aqueous supply. Such devices may be combined with other water treatment devices, or may be placed inline with one or more additional water purifying devices as part of an apparatus such as a water purificationsystem, a wastewater or sewage treatment

system, or any system designed to remove or reduce the concentration of inorganic and/or organic compounds in an aqueous solution.

Also disclosed is an apparatus for denitrifying an aqueous solution. The apparatus generally consists of one or more devices, each device comprising a culture of one or more autotrophic bacteria, an Fe(0) composition, and a container means for contacting the solution with the bacteria in the presence of the Fe(0) composition in such a device. Exemplary apparatus include sequencing batch reactor, a continuous culture system, a water treatment plant, a sewage treatment facility, a water purifying system, a wastewater treatment facility, or a detoxification system for aqueous solutions.

A further aspect of the invention is a semipermeable reactive barrier used for denitrifying groundwater in situ. This device generally consists of an in-ground barrier onto which a culture of one or more autotrophic bacteria and an Fe(0) composition is provided. The groundwater is in contact with the bacteria and the Fe(0) composition, such that the pollutants present in the water are remediated via the synergistic abiotic and biotic processes disclosed herein. Illustrative examples of compounds which may be remediated by such devices include nitrogen- or sulfur-containing compounds such as nitrate, nitrite, sulfate, and sulfite, and compounds containing one or more redox-sensitive metal ions such as mercury, strontium, technetium, silver, cesium, chromium, and uranium.

The present further provides compositions for use in the apparatus and devices used in situ and ex situ for the remediation of toxic compounds from aqueous environments. Such Fe(0)-bacterial compositions are useful in devices such as flow-through reactors, biofermenters, reactive barriers, packed or fluidized bed reactors, and the like which are useful in the practice of the methods disclosed herein.

The solid support used in the composition may be in the form of an apparatus or device which comprises a chamber, one or more inlet ports, one or more outlet ports, and a matrix within the chamber to which the Fe(0) and bacterial cells are in proximity. In an illustrative embodiment, the inventors passed a solution containing nitrate over a column containing Fe(0) and a culture of autotrophic denitrifying bacteria, and nitrate was removed from solution via the combined biotic/abiotic processes which occurred in the flow-through bioreactor.

3.0 BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. **1**. An example of a treatment wall using a ditch configuration (from Vidic and Pohnand, 1996).

FIG. **2**. An example of an injected treatment zone (from Vidic and Pohnand, 1996).

FIG. **3**. An example of a funnel and gate system (from Vidic and Pohnand, 1996).

FIG. **4**A. CT degradation with concomitant CF production (after Weathers and Parkin, 1995).

FIG. **4**B. CT degradation with concomitant CF production and subsequent degradation (after Weathers and Parkin, 1995). The degradation process is faster and more complete with the Fe(0)-methanogen combination.

FIG. **5**. Chlorinated methanes remaining after 68 h. About one-half of the added CT (200 nm) was converted to dechlorinated products (e.g., formate, CO, and CO_2). Product distribution changed significantly with more complete dechlorination with the iron-methanogen combination.

FIG. **6**. Removal of CF in steel-wool columns (after Weathers et al., 1997). The iron-methanogen combination consistently removes more CF, and the process appears to be sustainable.

FIG. **7**. Effluent methane concentration in steel-wool columns (after Weathers et al., 1997). A healthy population of methanogens was established while removing 1.5 μM of CF.

FIG. **8**. Laboratory apparatus for flow-through aquifer columns.

FIG. **9**A–FIG. **9**B. FIG. **9**A. Autotrophic growth of *P. denitrificans* ATCC 17741 in a dual flask apparatus was coupled to Fe(0) corrosion and nitrate reduction. FIG. **9**B. Flask A contained metallic iron and water, and flask B contained nitrate-amended, carbonate-buffered medium inoculated with bacteria

FIG. **10**A. CF degradation in batch reactors containing 2 g of iron filings and killed or live cells, or mineral medium. Filled symbols indicate treatments that were not amended with iron. All incubations received CF except those designated CF free.

FIG. **10**B. DCM formation in batch reactors containing 2 g of iron filings and killed or live cells, or mineral medium. Filled symbols indicate treatments that were not amended with iron. All incubations received CF except those designated CF free.

FIG. **10**C. Hydrogen evolution and utilization in batch reactors containing 2 g of iron filings and killed or live cells, or mineral medium. Filled symbols indicate treatments that were not amended with iron. All incubations received CF except those designated CF free.

FIG. **10**D. Methane production in batch reactors containing 2 g of iron filings and killed or live cells, or mineral medium. Filled symbols indicate treatments that were not amended with iron. All incubations received CF except those designated CF free.

FIG. **11**. CF degradation and DCM production in batch reactors amended with hydrogen.

FIG. **12**A. Hydrogen evolution and utilization in batch reactors containing 2 g of iron filings. None, inhibitor-free control; BESA, 50 mM BESA; Van, 100 mg/L vancomycin; BESA+Van, 50 mM BESA and 100 mg/L vancomycin.

FIG. **12**B. Methane production in batch reactors containing 2 g of iron filings. None, inhibitor-free control; BESA, 50 mM BESA; Van, 100 mg/L vancomycin; BESA+Van, 50 mM BESA and 100 mg/L vancomycin

FIG. **13**A. Hydrogen evolution and utilization in batch reactors amended with hydrogen. DI, deionized water control; None, inhibitor-free control; BESA, 50 mM BESA; Van, 100 mg/L vancomycin; BESA+Van, 50 mM BESA and 100 mg/L vancomycin.

FIG. **13**B. Methane production in batch reactors amended with hydrogen. DI, deionized water control; None, inhibitor-free control; BESA, 50 mM BESA; Van, 100 mg/L vancomycin; BESA+Van, 50 mM BESA and 100 mg/L vancomycin.

FIG. **13**C. CF degradation in batch reactors amended with hydrogen. DI, deionized water control; None, inhibitor-free control; BESA, 50 mM BESA; Van, 100 mg/L vancomycin; BESA+Van, 50 mM BESA and 100 mg/L vancomycin.

FIG. **14**A. CF concentrations in the column reactor effluents. The average influent CF concentration was 1.61±0.49 μM, and the average effluent CF concentration in the glass bead control column was 1.29±0.85 μM.

FIG. **14**B. Methane concentrations in the column reactor effluents. The average influent CF concentration was 1.61±0.49 μM, and the average effluent CF concentration in the glass bead control column was 1.29±0.85 μM.

FIG. **15**A. Permeable barrier for groundwater remediation.

FIG. **15**B. Plan view of the permeable barrier for groundwater remediation. Shown is **101** Contaminated water, **102** Aquifer, **103** Groundwater, **104** Trench, **105** well and **106** iron and bacteria reactive wall.

FIG. **15**C. Profile view of the permeable barrier.

FIGS. **16**A–**16**D. Example of a sequencing batch reactor. FIG. **16**A Fill (i) Deoxygenate, FIG. **16**B Anaerobic Sitr (ii), FIG. **16**C Settle (iii), FIG. **16**D Decant (iv), FIG. **16**E Idle, Cycle Adjustment and Waste Sludge (v).

FIG. **17**. Example of a fluidized bed reactor.

FIG. **18**. Example of flow-through packed column device of the present invention. The column contains a composition comprising Fe(0) and autotrophic bacteria.

4.0 DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The present invention overcomes limitations in the prior art such as those which rely on exclusively biotic or exclusively abiotic processes. For example, the invention is superior to the expensive "green rust" process in two critical areas: 1) the end product of "green rust" reduction is ammonium ion, which is toxic and pollutes the water source; and 2) "green rust" processes have remediation rates which are unsuitable for commercial-scale flow-through reactors and in situ remediation of leachates.

In contrast, the present invention provides gaseous (volatile) end products which are dissipated from the aqueous solution or environmental site, and as such, the compositions of the invention do not foul or pollute a site where it is employed. Likewise, the remediation rates afforded by the present compositions and methods are substantially higher than those afforded by "green rust" processes, and are therefore useful in commercial application of in situ or inline bioremediation. Moreover, the invention provides an important economical improvement over the existing art, by utilizing inexpensive, readily-available Fe(0), rather than relying on expensive or scarce components such as "green rust."

The invention further provides improvements over the prior art processes which require extraneous addition of hydrogen gas to fuel systems which rely on purely biotic processes such as those methods involving hydrogen-utilizing bacteria. A major shortcoming of these prior art methods is that large-scale H_2 delivery is neither cost-effective nor readily accomplished. H_2 gas is only slightly soluble in water and its hazardous (and explosive) properties make handling and storage problematic and often prohibitively expensive. The present invention overcomes these limitations by providing a process in which the production of H_2 is accomplished by the concomitant corrosion of Fe(0) substrates in solution. This invention overcomes these and other prior-art limitations by providing a composition which comprises Fe(0) and a culture of one or more hydrogenotrophic bacteria to permit the development of remediation processes which exploit favorable biogeochemical interactions.

The present invention further overcomes limitations of prior art systems which utilize methanol, a common electron donor, to support autotrophic biotic denitrification. The stoichiometric requirement for the metabolism of methanol and nitrate is 2.5 mg CH_3OH/mg NO_3—N. The diffusion coefficient of methanol is one-half that of nitrate, so in order to prevent methanol from being flux limiting in biofilm systems, it must be fed at twice the stoichiometric requirement (i.e. at 5 mg CH_3OH/mg NO_3—N) (Williamson and McCarty, 1976). This excess methanol has detrimental side-effects on water quality, such as the production of excess biomass and the formation of trihalomethanes. Moreover, methanol can cause blindness. Such side-effects are absent in the present invention through the use of Fe(0) as a source for the production of hydrogen gas. Hydrogen gas offers a second advantage over prior art methods, due to its higher diffusivity in aqueous media. Therefore, the accumulation of toxic compounds such as trihalomethane, and the need for disinfecting excess biomass is eliminated when employing the processes of the present invention.

4.1 Definitions

The following words and phrases have the meanings set forth below:

a, an: In keeping with the well-established precedent in patent law, the use of the articles "a" and "an" are intended in all instances to mean "one or more."

Expression: The combination of intracellular processes, including transcription and translation undergone by a coding DNA molecule such as a structural gene to produce a polypeptide.

Promoter: A recognition site on a DNA sequence or group of DNA sequences that provide an expression control element for a structural gene and to which RNA polymerase specifically binds and initiates RNA synthesis (transcription) of that gene.

Structural gene: A gene that is expressed to produce a polypeptide.

Transformation: A process of introducing an exogenous DNA sequence (e.g., a vector, a recombinant DNA molecule) into a cell or protoplast in which that exogenous DNA is incorporated into a chromosome or is capable of autonomous replication.

Transformed cell: A cell whose DNA has been altered by the introduction of an exogenous DNA molecule into that cell.

Transgenic cell: Any cell derived or regenerated from a transformed cell or derived from a transgenic cell.

Vector: A DNA molecule capable of replication in a host cell and/or to which another DNA segment can be operatively linked so as to bring about replication of the attached segment. A plasmid is an exemplary vector.

4.2 Halocarbon Contamination

Fe(0) will enhance microbial activity by serving as electron donor (via water-derived hydrogen, produced by cathodic depolarization) to support hydrogenotrophic growth and reductive biotransformations. Bacterial viability may be further enhanced by abiotic removal of toxic compounds by Fe(0) below toxic levels. The bacteria, in turn, might enhance the abiotic process by removing the passivating (cathodic) hydrogen layer from the Fe(0) surface and by further degrading some products of reductive treatment with Fe(0). Chlorinated aliphatic hydrocarbons (CAHs) are frequent groundwater contaminants (Riley et al., 1992).

They are susceptible to biotransformations catalyzed by pure and mixed methanogenic (Bouwer et al., 1981; Bouwer and McCarty, 1983; Gossett, 1985; Egli et al., 1987; Krone et al., 1989a; Mikesell and Boyd, 1990; Bagley and Gossett, 1995; Hughes and Parkin, 1996) and nonmethanogenic, anaerobic cultures (Egli et al., 1987; Egli et al., 1990; Egli et al., 1988; Galli and McCarty, 1989; Fathepure and Tiedje, 1994). Electron donors that have been investigated for methanogenic biotransformation of CAHs include methanol (Mikesell and Boyd, 1990; Bagley and Gossett, 1995), acetate (Bouwer et al., 1981; Bouwer and McCarty, 1983; Hughes and Parkin, 1996), and hydrogen (DiStefano et al., 1992). Hydrogen is a more thermodynamically favorable electron donor than either methanol or acetate (Zinder, 1993), and its high diffusivity through biofilms ensures that the target CAHs would be flux-limiting to optimize removal rates. Nevertheless, practical problems exist with regard to the use of hydrogen due to its low solubility in water, about 1.6 mg/L at 20° C., based on a Henry's constant of 6.83×10^{4} atm/mol (Metcalf and Eddy, Inc., 1991).

One way that hydrogen can be introduced into aqueous solution involves the use of iron metal. When Fe^0 is immersed in anoxic water, hydrogen is produced (Reardon, 1996):

$$Fe^0+2H_2O\rightarrow Fe^{2+}+2OH^-+H_2 \qquad \text{(Equation 16)}$$

Pure cultures of methanogenic (Daniels et al., 1987; Rajagopal and LeGall, 1989; Belay and Daniels, 1990; Lorowitz et al., 1992), homoacetogenic (Rajagopal and LeGall, 1989), and sulfate-reducing bacteria (Rajagopal and LeGall, 1989) have demonstrated the ability to use cathodic hydrogen as an energy source for growth in short-term batch studies. Also, anaerobic bacteria are commonly associated with the corrosion of metals (Hamilton, 1985; Lee et al., 1995). However, the ability of methanogens to sustain growth on cathodic hydrogen in the presence of CAHs has not been established. This is particularly important because many CAHs, especially chloroform (CF), extremely inhibitory to methanogens (Hughes and Parkin, 1996; Bauchop, 1967; Thiel, 1969; Swanwick and Foulkes, 1971; Prins et al., 1972; Yang and Speece, 1986; Hickey et al., 1987).

The biotransformation of CAHs using cathodic hydrogen as electron donor is confounded by the direct, abiotic reduction of CAHs by Fe^0 (Gillham and O'Hannesin, 1994; Matheson and Tratnyek, 1994; Helland et al., 1995; Orth and Gillham, 1995). In the presence of 100-mesh iron powder, carbon tetrachloride (CT) underwent sequential dehalogenation to CF and then to dichloromethane (DCM), which was not further degraded (Matheson and Tratnyck, 1994).

Thus it is well-known that anaerobic bacteria can catalyze the reductive dechlorination of CAHs, that CAHs can be transformed abiotically using Fe^0, and that Fe^0 can support the growth of anaerobic bacteria. Furthermore, there are limitations of the biotic process because of potential toxicity of parent CAHs and their metabolites and to the abiotic process because of the potential buildup of "dead-end" products. It is hypothesized that the presence of an active methanogenic consortium in the presence of Fe^0 may enhance the rate and extent of degradation of selected CAHs.

4.3 Sr(II), Cs(I), Cr(VI) and U(VI) Reduction

Pure cultures of methanogenic, homoacetogenic, sulfate-reducing, and denitrifying bacteria have demonstrated the ability to use cathodic hydrogen (reaction 3 above) as an energy source for growth in short-term, batch studies (Daniels et al., 1987; Rajagopal and LeGall, 1989; Till et al., 1998). As noted, such organisms have also been shown to facilitate reductive dechlorination and reduction of Cr(VI) and U(VI).

The present invention improves upon these results by providing methods for the remediation of a variety of toxic ions using the bioaugmented Fe(0) compositions disclosed herein. These methods improve both the rate and extent of overall contaminant removal when compared to solely biotic based methods. In particular, compounds containing Cs(I), Sr(II, Cr(VI), and U(VI) are contemplated by the inventors to be remediable using the disclosed compositions and methods. These combined biotic-abiotic processes have a number of advantages in addition to the removal of the parent compounds. For example, abiotic removal of Cs(I), Sr(II), Cr(VI) and/or U(VI) from solution or soils reduces the toxicity of these metals to the microbes, and abiotic removal of the chlorinated compounds may reduce their concentrations to levels that are not toxic to the microbes. The microorganisms, in turn, may further degrade dead-end products that accumulate during abiotic transformation (e.g., CF and DCM from CT).

4.4 Iron-Supported Autotrophic Denitrification (ISAD)

ISAD is an improvement over existing biological nitrate removal processes because the autotrophic organisms used in this process grow on hydrogen gas produced by the corrosion of zero-valent iron by water (i.e., "cathodic hydrogen", H_2, is derived from water, H_2O). Hydrogen gas is thermodynamically a more favorable reductant and has a greater diffusivity through biofilms compared to the organic substrates that are commonly used to support biological denitrification (e.g., methanol and acetate). While these properties are conducive to enhanced nitrate removal, previous use of hydrogen as a substrate to support denitrification has been limited by its low solubility and its hazardous (explosive) properties during handling and storage. These limitations are overcome by using Fe(0) to continuously generate cathodic hydrogen to support autotrophic dentrification. In this process, Fe(0) is also used as a direct reductant to remove nitrates abiotically. In addition, autotrophic bacteria use CO_2 as carbon source to synthesize new cell tissue and thus have lower biomass production that the bacteria used in conventional denitrification systems fed organic substrates. Thus, adverse side-effects on water quality due to residual organic compounds and excessive biomass production are eliminated.

4.5 Organisms Useful in the Practice of the Invention

Mixed cultures of autotrophic, hydrogen-oxidizing, denitrifying bacteria are often found in groundwater systems. Smith et al. (1994) isolated a mixed culture of hydrogenotrophic denitrifiers from a nitrate-contaminated sand and gravel aquifer in Cape Cod, Mass. Nine strains of hydrogen-oxidizing, denitrifying bacteria were isolated from the aquifer with all of the strains able to grow heterotrophically as well. None of the strains were similar to *P. denitrificans*.

P. denitrificans is one of the most intensively studied denitrifying microorganism due to its nutritional versatility (Mateju et al., 1992). Its well-known capability to reduce nitrogenous compounds with hydrogen is the basis of several approaches for denitrification of drinking water (Dries et al., 1988; Gros et al., 1988; Kurt et al., 1987). Using

hydrogen as an oxidizable substrate for water treatment offers a number of important benefits, such as food-grade quality of the reductant, process reliability, low excess sludge production, and no need for intensive monitoring or biological post-treatment to control and remove residual reductant (Selenka and Dressler, 1990). The main disadvantages of using hydrogen in water treatment involve its low solubility and delivering difficulty, and the slow growth rate of autotrophic bacteria using carbon dioxide as their sole carbon source (Dries et al., 1988).

Few bacterial strains are known to completely denitrify with hydrogen gas as the sole energy source. Evidence exists that a consortium of bacteria is often responsible for hydrogenotrophic denitrification. A list of several microorganisms isoalted from and identified in hydrogenotrophic, denitrifying biofilms is show in Table 1. Liessens et al. (1992) isolated a large diversity of organisms from a hydrogenotrophic denitrification reactor. Acinetobacter sp., Aeromonas sp., Pseudomonas sp., and *Shewanella putrefaciens* were repeatedly isolated from the hydrogenotrophic sludge. In a hydrogenotrophic denitrification reactor at Rasseln, Germany, Selenka and Dressler (1990) characterized the chemolithotrophic bacteria present in the plant. They found bacterial strains as representatives of the genera Pseudomonas, Alcaligenes, and Achromobacter. The opportunistic presence of Acinetobacter spp. unable to reduce nitrate in the plant was unclear, although these species may be involved in creating anoxic conditions (Liessens et al., 1992).

Several genera of hydrogenotrophic bacteria have been identified by the inventors to be useful in the preparation of zero-valent iron-supported cultures for remediation of a variety of contaminants and pollutants. In particular, species of bacteria selected from the group consisting of Acetobacterium spp., Achromobacter spp., Aeromonas spp., Acinetobacter spp., Aureobacterium spp., Bacillus spp., Comamonas spp., Dehalobacter spp., Dehalospirillum spp., Dehalococcoide spp., Desulfurosarcina spp., Desulfomonile spp., Desulfobacterium spp., Enterobacter spp., Hydrogenobacter spp., Methanosarcina spp., Pseudomonas spp., Shewanella spp., Methanosarcina spp., Micrococcus spp., and Paracoccus spp are particularly preferred for the practice of the invention. These bacterial compositions may include one or more strains of bacteria selected from the group consisting of *Acetobacterium woodi, Aeromonas hydrophila, Aeromonas sobria, Alcaligenes eutrophus, Comamonas acidovorans, Dehalococcoide restrictus, Dehalococcoide multivorans, Dehalococcoide ethenogene, Desulfobacterium tiedje, Enterobacter agglomerans, Hydrogenobacter thermophilus, Methanosarcina barkeri, Methanosarcina mazei, Methanosarcina thermophila, Paracoccus denitrificans, Pseudomonas aureofaciens, Pseudomonas maltophilia, Pseudomonas mendocina,* and *Shewanella putrefaciens.* Alternatively, hydrogenotrophic bacteria present in anaerobic sludge or anaerobic sediments may also be used in the practice of the invention, and may be combined with one or more pure cultures of hydrogenotrophic bacteria for the development of compositions comprising mixed microbial cultures in combination with zero-valent iron.

4.6 Hydrogenotrophic Denitrifiers

Since denitrification is a respiratory process, an oxidizable substrate or electron donor is required. Most of the research and work done in the area of denitrification has been done with heterotrophic bacteria that utilize organic compounds. The present invention focuses on autotrophic bacteria that are characterized by their ability to obtain energy by the oxidation of gaseous hydrogen (H_2) via anaerobic respiration with nitrate and synthesize cell material by the reductive assimilation of carbon dioxide (CO_2) via the Calvin cycle.

TABLE 1

MICROORGANISMS FROM HYDROGENOTROPHIC, DENITRIFYING BIOFILMS

Bacteria	Location	Reference
Achromobacter sp.	Denitrification reactor	Selenka and Dressler, 1990
Acinetobacter sp.	Denitrification reactor	Liessens et al., 1992
Aeromonas hydrophila	Denitrification reactor	Liessens et al., 1992
A. sobria	Denitrification reactor	Liessens et al., 1992
Alcaligenes sp.	Denitrification reactor	Selenka and Dressler, 1990
Alcaligenes eutrophus	Denitrification reactor	Dries et al., 1988
Aureobacterium sp.	Denitrification reactor	Liessens et al., 1992
Bacillus sp.	Denitrification reactor	Liessens et al., 1992
Comamonas acidovorans	Denitrification reactor	Liessens et al., 1992
Hydrogenobacter thermophilus	Denitrification reactor	Liessens et al., 1992
Micrococcus sp.	Denitrification reactor	Liessens et al., 1992
Mixed culture	Oligotrophic aquifer	Smith et al., 1994
Paracoccus denitrificans	Denitrification reactor	Liessens et al., 1992
Pseudomonas aureofaciens	Denitrification reactor	Liessens et al., 1992
P. maltophilia	Denitrification reactor	Dries et al., 1988
P. mendocina	Denitrification reactor	Liessens et al., 1992
P. putrefaciens	Denitrification reactor	Dries et al., 1988
P. stutzeri	Denitrification reactor	Liessens et al., 1992
P. syringae	Denitrification reactor	Liessens et al., 1992
Serratia odorifera	Denitrification reactor	Liessens et al., 1992
Shewanella putreficans	Denitrification reactor	Liessens et al., 1992
Thiobacillus denitrificans	Denitrification reactor	Lewandowski et al., 1987

Hydrogen metabolism is widely distributed among various well-described physiological groups of bacteria, such as the methanogenic and acetogenic bacteria, the sulfate-reducers, the organotrophic fermentative bacteria, or the N_2-fixing bacteria (Madigan et al., 1987). Hydrogen is also reported to be a driving mechanism for the microbial degradation of micropollutants, such as atrazine, in soils and in drinking water (Wierinck et al., 1990). Aerobic hydrogen bacteria belong to different taxonomic groups and are all facultative autotrophs with one exception, *Hydrogenobacter thermophilus,* which is an obligate autotroph. The hydrogen bacteria all show versatile metabolism; many of them, especially those regarded as Pseudomonas and Alcaligenes are characterized by the ability to use a wide variety of carbon substrates (Mateju et al., 1992). So far, relatively few bacteria have been found that are able to denitrify nitrate, nitrite, or nitrous oxide under hydrogenotrophic conditions (Liessens et al., 1992). Hydrogen-utilizing, denitrifying bacteria take up hydrogen gas by using two hydrogenase enzymes. The first enzyme is membrane bound and used in the production of ATP. The second enzyme is cytoplasmic (soluble) and directly reduces NAD^+ to NADH for autotrophic growth via the Calvin cycle (Madigan et al., 1997). It can be seen from the following equation that the theoretical consumption of hydrogen is 0.35 mg H_2 per 1 mg NO3—N reduced to dinitrogen gas (N_2):

$$2NO_3^- + 5H_2 + 2H^+ \rightarrow N_2 + 6H_2O$$

$$\Delta G_0 = -565.96 \text{ kJ}$$

Experimental H_2 requirements have been shown to be slightly higher than the stoichiometric values and range from 0.38 to 0.40 mg H_2 per 1 mg NO_3—N reduced to N_2 (Gros et al., 1988; Dries et al., 1988).

4.7 Methods of Nucleic Acid Delivery and DNA Transfection

In certain embodiments, it is contemplated that nucleic acid segments encoding one or more of the bacterial enzymes required for the detoxification of one or more of the target pollutants may be cloned and used to transfect appropriate host cells to provide genetically engineered recombinant microorganisms with enhanced activity for bioremediation of particular contaminants. Technology for introduction of nucleic acids into cells is well-known to those of skill in the art. Four general methods for delivering a nucleic segment into cells have been described:

(1) chemical methods (Graham and Van Der Eb, 1973);

(2) physical methods such as microinjection (Capecchi, 1980), electroporation (Wong and Neumann, 1982; Fromm et al., 1985) and the gene gun (Yang et al., 1990);

(3) viral vectors (Lu et al., 1993; Eglitis and Anderson, 1988); and

(4) receptor-mediated mechanisms (Curiel et al., 1991; Wagner et al., 1992).

In a preferred embodiment, the engineering of recombinant autotrophic bacterial cells to contain DNA segments encoding modified denitrifying enzymes is desirable for improved denitrifying bioreactors. In such an embodiment, a mutant nucleic acid is preferable in which the oxygen repression of the genes has been lessened or eliminated.

4.8 Recombinant Host Cells and Vectors

Particular aspects of the invention concern the use of plasmid vectors for the cloning and expression of various genes encoding detoxifying enzymes such as those involved in bioremediation of pollutants, and in particular, denitrification by autotrophic bacteria. The generation of recombinant vectors, transformation of host cells, and expression of recombinant proteins is well-known to those of skill in the art. Prokaryotic hosts are preferred for such expression, with those organisms listed in Table 1 being particularly preferred.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells.

Those promoters most commonly used in recombinant DNA construction include the β-lactamase (penicillinase) and lactose promoter systems (Chang et al., 1978; Itakura et al., 1977; Goeddel et al., 1979) or the tryptophan (trp) promoter system (Goeddel et al., 1980). The use of recombinant and native microbial promoters is well-known to those of skill in the art, and details concerning their nucleotide sequences and specific methodologies are in the public domain, enabling a skilled worker to construct particular recombinant vectors and expression systems for the purpose of producing recombinantly engineered autotrophic bacterial host cells of the present invention.

4.9 Site-Specific Mutagenesis

Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique, well-known to those of skill in the art, further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Such mutations are particularly desirable in generating mutant strains of autotrophic bacteria capable of detoxifying the pollutants of interest with improved properties or greater efficiency than native, unengineered bacterial cell lines.

Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 14 to about 25 nucleotides in length is preferred, with about 5 to about 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by various publications. As will be appreciated, the technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage are readily commercially-available and their use is generally well-known to those skilled in the art. Double-stranded plasmids are also routinely employed in site directed mutagenesis which eliminates the step of transferring the gene of interest from a plasmid to a phage.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector or melting apart of two strands of a double-stranded vector which includes within its sequence a DNA sequence which encodes the desired peptide. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically. This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected peptide-encoding DNA segments using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting as there are other ways in which sequence variants of peptides and the DNA sequences encoding them may be obtained. For example, recombinant vectors encoding the desired peptide sequence may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants. Specific details regarding these methods and protocols are found in the teachings of Maloy et al., 1994; Segal, 1976; Prokop and Bajpai, 1991; Kuby, 1994; and Maniatis et al., 1982, each incorporated herein by reference, for that purpose.

4.10 Biological Functional Equivalents

In certain embodiments, the inventors contemplate the preparation of engineered microorganisms having the ability to detoxify water and wastewater contaminants using the methods disclosed herein. In one embodiment, the inventors contemplate the transformation of autotrophic bacterial cultures, such as those listed in Table 2, to provide "second generation" denitrifying cell lines which have improved

detoxification properties. Modification and changes may be made in the structure of the enzymes (or regulatory proteins) involved in this detoxification to provide such recombinantly engineered host cells. Such modifications would still lead to the creation of a host cell which encodes the necessary proteins, enzymes, or peptides to perform such biotic bioremediation.

The following is a discussion based upon changing the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule. The amino acid changes may be achieved by changing the codons of the DNA sequence, according to Table 2.

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the peptide sequences of the disclosed compositions, or corresponding DNA sequences which encode said peptides without appreciable loss of their biological utility or activity.

TABLE 2

Amino Acids			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporate herein by reference). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte and Doolittle, 1982), these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (−0.4); threonine (−0.7); serine (−0.8); tryptophan (−0.9); tyrosine (−1.3); proline (−1.6); histidine (−3.2); glutamate (−3.5); glutamine (−3.5); aspartate (−3.5); asparagine (−3.5); lysine (−3.9); and arginine (−4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, i.e., still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred. It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Pat. No. 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U.S. Pat. No. 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0±1); glutamate (+3.0±1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (−0.4); proline (−0.5±1); alanine (−0.5); histidine (−0.5); cysteine (−1.0); methionine (−1.3); valine (−1.5); leucine (−1.8); isoleucine (−1.8); tyrosine (−2.3); phenylalanine (−2.5); tryptophan (−3.4). It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

4.11 Means for Preparing Site-Directed Mutagenized Enzymes

In certain embodiments, engineered microorganisms may be developed to enhance the rate or specificity of pollutant detoxification by the autotrophic microorganisms. In particular, enzymes responsible for bacterial degradation of target pollutants, and the underlying DNA sequences which encode them may be mutagenized to improve performance of the biotic processes. As such, the invention concerns the preparation of mutants using recombinant DNA methodologies which are known to those of skill in the art.

Nucleic acid segments encoding degradative enzymes may be subjected to site directed mutagenesis to prepare variants having improved remediation properties as described above. One preferred method for the mutagenesis of such polynucleotides is the PCR™-based strand overlap extension (SOE) (Ho et al., 1989) method. The techniques of PCR™ are well-known to those of skill in the art, as described hereinabove. The SOE procedure involves a two-step PCR™ protocol, in which a complementary pair of internal primers (B and C) are used to introduce the appropriate nucleotide changes into the wild-type sequence. In two separate reactions, flanking PCR™ primer A (restriction site incorporated into the oligo) and primer D (restriction site incorporated into the oligo) are used in conjunction with

primers B and C, respectively to generate PCR™ products AB and CD. The PCR™ products are purified by agarose gel electrophoresis and the two overlapping PCR™ fragments AB and CD are combined with flanking primers A and D and used in a second PCR™ reaction. The amplified PCR™ product is agarose gel purified, digested with the appropriate enzymes, ligated into an expression vector, and transformed into *E. coli* JM101, XL1-Blue™ (Stratagene, La Jolla, Calif.), JM105, or TG1 (Carter et al., 1985) cells. Clones are isolated and the mutations are confirmed by sequencing of the isolated plasmids.

4.12 Means for Expressing Enzymes Using Recombinant Vectors

In many cases, it may be desirable to engineer the disclosed microorganisms to improve the detoxification activity of the cells in situ. A particular aspect of the present invention is the production of recombinant degradative enzymes in large quantity. Such methods are well-known to those of skill in the art, and have been described in detail hereinabove. To overexpress one or more of the enzymes necessary for the detoxification of environmental contaminants, DNA fragments encoding the appropriate polypeptides may be cloned into a variety of expression vectors. Such vectors may contain a multiple restriction enzyme cloning site that situates the nucleic acid segment of interest such that its expression is controlled from an inducible promoter. Methods for determining orientation of the inserted segment, induction of the promoter, growth conditions, and restriction enzyme analysis, and recovery of the produced protein are well-known to those of skill in the art. Expression and quantitation of the polypeptides are determinable via standard methods such as SDS-PAGE, Western blot analysis, and protein determination assays.

5.0 EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

5.1 Example 1

Bioaugmentation of Fe(0) Barriers

The inventors have shown that combining Fe(0) with an active methanogenic population can enhance the removal of chlorinated aliphatic compounds. Batch serum bottle assays with iron powder, iron filings, or steel wool were used to investigate the potential of the iron-methanogen treatment process. Flow-through column reactors packed with steel wool were also used. Systems were seeded with a methanogenic enrichment culture or with pure cultures of *Methanosarcina barkeri* or *Methanosarcina thermophila*. The transformation of carbon tetrachloride (CT) and its dechlorinated homologs, chloroform (CF) and dichloromethane (DCM), are described in this example.

The iron-methanogen combination resulted in higher rates of removal and more complete dechlorination. FIG. **4A** shows that CT removal is faster when both Fe(0) and the methanogenic enrichment are together. The effect is even more pronounced with CF, which is produced from hydrogenolysis of CT and is subsequently removed much with the iron-methanogen combination (FIG. **4B**). Product distribution is also better for the iron-methanogen combination, which exhibited more complete dechlorination (FIG. **5**). These data and other studies have shown that when the methanogenic enrichment is present, the predominant product is DCM while CF is the dominant product with Fe(0) alone. With acclimation (5–10 days), DCM is subsequently biodegraded readily. Preliminary studies with pure cultures of *Methanosarcina barkeri* and *Methanosarcina thermophila* have shown that there may be a pathway shift (from hydrogenolysis towards reductive hydrolysis) when Fe(0) is bioaugmented; more $^{14}CO_2$ is formed when the bacteria and Fe(0) are incubated together.

Pseudo-first-order rate coefficients were determined in a different set of studies to evaluate the kinetic advantage of the iron-methanogen combination. For both carbon CT and CF, decay rate coefficients were significantly higher for the iron-methanogen combination than for the iron-only, bacteria-only, and iron-killed bacteria systems (Table 3). The rate coefficient for the combined Fe(0) and live cell treatment was 2.4 times the sum of the rate coefficients for the separate treatments containing Fe(0) or live cells. Hence, abiotic and microbial CF degradation processes were not independent when Fe(0) and cells were combined in a single reactor. This synergism was attributed to the production of cathodic (water-derived) hydrogen as a result of the oxidation of Fe(0). This increased the availability of primary substrates available for CF cometabolism.

TABLE 3

PSEUDO-FIRST-ORDER RATE COEFFICIENTS FOR CF

Treatment	Pseudo-first-order rate coefficient (day^{-1})
Fe(0) + live cells	0.72
Fe(0) + killed cells	0.20
live cells alone	0.10
Fe(0) alone	0.08

Column studies with CF and steel wool showed that the process is stable over time (FIG. **6**). Removal of CF in the steel-wool column seeded with a methanogenic enrichment was consistently higher than the steel-wool-only column. These data show that CF removal by anaerobic bacteria using H_2 as the sole electron donor is sustainable, an observation that was uncertain based on batch studies where methanogenesis was inhibited by the presence of CF. An active consortium of methane-producing organisms was established as evidenced by the increased production of methane (FIG. **7**).

It was shown that Fe(0) ablotically reduced nitrate to ammonium and that this process could be enhanced by the presence of autotrophic denitrifiers which reduce nitrate to more innocuous products (i.e., N_2O and N_2). Such results are particularly important for environmental sites such as uranium mill tailings, where waste nitric acid is a common contaminant.

It has also been shown that removal of chlorinated compounds such as carbon tetrachloride can be accomplished under denitrifying conditions with the production of more oxidized end products (CO_2) than usually seen under sulfate-reducing or methanogenic conditions (Criddle et al., 1990). Denitrifiers have also been shown to directly participate in the removal of Cr(VI) from the aqueous phase (Guan

et al., 1993). Thus, the invention provides a means for the combined synergistic processes that combine Fe(0) substrates and detoxifying autotrophic bacterial systems to remove target environmental pollutants.

Studies demonstrate that microbial-Fe(0) treatment systems offer significant advantages over approaches where either process is used alone to treat reducible contaminants such as chlorinated compounds, Cr(VI), U(VI), nitroaromatic compounds, and various agrochemicals. In a field-scale application, cells grown in a fermenter or sludge from an anaerobic digester could be injected into a permeable, iron-containing, reactive barrier similar to that proposed by Blowes et al. (1995). For some compounds, combined microbial-abiotic systems may accelerate the rate of transformation and extent of mineralization. Nevertheless, the proliferation of bacteria in an improperly designed reactive barrier could reduce the hydraulic conductivity of the barrier, thereby hindering the flow of groundwater through it.

Hydrogen produced from the corrosion of Fe(0) by H_2O serves as energy source for the microbes and as electron donor for the biotransformation of reducible contaminants. The present invention provides the first reported combination of iron-microbial consortia for remediating pollution in situ and ex situ.

Although H_2 is one of the most thermodynamically favorable substrates for anaerobic microbes, its use as electron donor for bioremediation purposes has been limited by its low solubility. The observation that cathodic H_2 can support reductive dechlorination and other reductive biotransformations by anaerobic bacteria has significant application.

5.1.1 Materials and Methods

5.1.1.1 Experimental Design

Batch aquifer microcosms have been used to evaluate the potential for anaerobic bacteria to enhance the removal of Cr(VI), U(VI), and TCE in the presence of Fe(0). In order to determine the effect of competing electron acceptors commonly found in the subsurface, Fe(0) bioaugmentation studies were conducted under four electron acceptor conditions: denitrifying, iron reducing, sulfate reducing, and methanogenic. For each condition, biological and abiotic transformation mechanisms are studied separately and interactively to evaluate potential synergistic effects and interaction mechanisms. Some microcosms were amended with Fe(0) alone, some with bacteria, and some received both bacteria and Fe(0). Positive controls for microbial activity consisted of Fe(0)-free microcosms fed H_2. Sterile controls without Fe(0) were included to discern biodegradation from other potential losses (Table 4).

To investigate potential synergistic or antagonistic substrate interactions, the transformation of the target compounds was studied when present together and separately. Differences in removal efficiencies and product distribution were evaluated statistically using Student's t-test at the 95% confidence level. These studies demonstrated the benefits of combining Fe(0) with anaerobic bioremediation to remove various contaminant combinations. The data also establish time scales for removal of the target contaminants below acceptable levels, and assesses the nature of the contaminant mixture and redox conditions and how they affect removal kinetics and product distribution.

TABLE 4

BATCH REACTOR SETS FOR EACH ELECTRON ACCEPTOR CONDITION

Set Number	Description	Number of Replicates
1	Abiotic reactor with Fe(0), amended with TCE + Cr(VI) + U(VI)	3
2	Abiotic reactor with Fe(0), amended with TCE	3
3	Abiotic reactor with Fe(0), amended with Cr(VI)	3
4	Abiotic reactor with Fe(0), amended with U(VI)	3
5	Resting cells (without Fe(0)) amended with TCE + Cr(VI) + U(VI)	3
6	Resting cells (without Fe(0)) amended with TCE	3
7	Resting cells (without Fe(0)) amended with Cr(VI)	3
8	Resting cells (without Fe(0)) amended with U(VI)	3
9	Biologically active (without Fe(0), fed H_2) amended with TCE + Cr(VI) + U(VI)	3
10	Biologically active (without Fe(0), fed H_2) amended with TCE	3
11	Biologically active (without Fe(0), fed H_2) amended with Cr(VI)	3
12	Biologically active (without Fe(0), fed H_2) amended with U(VI)	3
13	Bioaugmented Fe(0), amended with TCE + Cr(VI) + U(VI)	3
14	Bioaugmented Fe(0), amended with TCE	3
15	Bioaugmented with Fe(0), amended with Cr(VI)	3
16	Bioaugmented Fe(0), amended with U(VI)	3
17	Abiotic controls (without Fe(0), fed H_2), amended with TCE + Cr(VI) + U(VI)	3
	TOTAL	51

In one study, an objective was to determine the effect that Fe(0) had on the viability of various microbial populations (i.e., denitrifiers, iron reducers, sulfate reducers, and methanogens) exposed to Cr(VI), U(VI), and chlorinated solvent mixtures, and to evaluate the effect that bacteria have on the abiotic reduction of the target contaminants by Fe(0).

Another study determined how environmental factors and substrate interactions affected the efficiency of bioaugmented Fe(0) barriers to attenuate the migration of chlorinated solvents, radionuclide, and heavy metal mixtures under various electron acceptor conditions. Continuous flow aquifer columns were constructed to mimic in situ conditions of microbial and Fe(0) exposure to contaminant mixtures (Table 5). As was the case with microcosm studies, biological and abiotic processes were studied separately and interactively to analyze the synergistic effect of combining Fe(0) with microorganisms. Selected perturbations of the hydraulic regime and of the redox conditions were applied to evaluate how engineered manipulations affect removal efficiency and product distribution.

The ability of bioaugmented Fe(0) barriers to remove excess nitrate and sulfate from uranium mill tailings is also evaluated in these columns. The effect of microbial growth on hydrodynamic properties may also be evaluated using tracer studies.

TABLE 5

FLOW-THROUGH COLUMN SETS

Set Number	Description	Number of Replicates
1	Abiotic columns, packed with aquifer material + Fe(0), fed TCE + Cr(VI) + U(VI)	2
2	Biologically active, packed with aquifer material (no Fe(0)), fed TCE + Cr(VI) + U(VI)	2

TABLE 5-continued

FLOW-THROUGH COLUMN SETS		
Set Number	Description	Number of Replicates
3	Biologically active, packed with aquifer material + Fe(0), fed TCE + Cr(VI) + U(VI)	2
4	Controls, packed with aquifer material (no Fe(0)), fed bactericide + TCE + Cr(VI) + U(VI)	2
	TOTAL	8

5.1.1.2 Microcosm Preparation

Aquifer microcosms are prepared in triplicate with 200 mL of basal mineral medium and 50 g of aquifer material in 250-mL serum bottles. The medium is bicarbonate-buffered near pH 7. This provides bicarbonate for autotrophic growth and precludes confounding effects by phosphate buffers reacting with iron. Microcosms are purged continuously with N_2/CO_2 (80/20 vol./vol.) to remove dissolved oxygen. Prior to capping them with Mininert valves, microcosms are amended with either Master Builder→Fe(0) filings (15% by weight) (Orth and Gillham, 1996), anaerobic bacteria, or both. The anaerobic bacteria are enriched from soil and sewage sludge under appropriate electron acceptor conditions. Sterile controls are autoclaved and poisoned with $HgCl_2$ (200 mg/l). No treatment controls (without Fe(0) or bacteria addition) are also prepared to obtain a baseline for comparing the effectiveness of different treatments. All microcosms are incubated quiescently in the dark at 20° C.

The basal medium provides inorganic nutrients for microbial growth (e.g., NH_4^+, trace metals, and the respective electron acceptor(s)) and contains various combinations of target contaminants (Table 3). Initial concentrations are representative of those found at natural sites. Levels of TCE contamination have been reported as 0.0002–870 mg/L in groundwaters and 0.0002–12,000 mg/kg in soils and sediments; levels of chromium contamination have been reported as 0.004–9 mg/L in groundwater and 1–6,900 mg/kg in soils and sediments; and levels of uranium contamination have been reported as 0–11,700 mg/L in groundwater and 0.0002–16 mg/kg in soils and sediments (Riley et al., 1992).

Electron acceptors, H_2, CH_4, Cr(VI), Cr(III), U(VI), U(IV), TCE and its dechlorinated homologues are monitored over time in each microcosm to compare lag periods and biodegradation rates for different treatment sets.

The lag period is determined as the time during which contaminant concentrations remain constant. Pseudo zero-order rates can be estimated as the ratio of contaminant removed (corrected for appropriate controls) to the corresponding time after the lag period. This information is used to statistically evaluate the ability of different treatments and mixtures to stimulate (or inhibit) the degradation of individual contaminants.

5.1.13 Aquifer Columns

Eight glass columns,(58 cm long, 2.2 cm ID) equipped with lateral sampling ports are packed with aquifer material as described by Siegrist and McCarty (1987) (FIG. **8**). One pair ("treatment" set) is packed with a mixture of aquifer material and Master Builder→Fe(0) filings (15% by weight) (Orth and Gillhan, 1996) and is seeded with anaerobic microorganisms. Two pairs ("no-treatment" sets) are packed with either seeded aquifer material alone or a aquifer material with Fe(0) filings alone; and a fourth pair ("sterile control" set) is packed with aquifer material and autoclaved for 4 h at 120° C. The feed solution to the sterile set also contains a bactericide ($HgCl_2$ at 200 mg/L). Cr(VI), U(VI), and TCE is initially fed at 10 mg/l (each). Influent analogue concentrations are based on the findings of the aquifer slurry studies. Columns are fed in an upflow mode at 2 ml/h (about 0.5 ft/day superficial velocity) using Harvard Model 22 syringe pumps equipped with 100 ml gas-tight glass and Teflon syringes (FIG. **9**). Column effluents are analyzed regularly for residual target contaminants, pH, dissolved oxygen, nitrate, nitrite, sulfate, ferrous iron, and methane. A Microtox→system is used to evaluate the efficacy of different columns in reducing the effluent toxicity.

Bromide tracer studies are performed at the beginning and end of the study to determine changes in liquid detention time, pore volume, and dispersion in the packed columns. A bromide solution (50 mg/l) is continuously injected at 2 ml/h. Effluent bromide concentrations are monitored until they reach influent levels. The mean liquid detention time is estimated as the time required for the effluent bromide concentration to reach 50% of the influent concentration. This time is multiplied by the flow rate in order to estimate the pore volume of the aquifer column. Porosity is calculated as the ratio of pore volume to the total volume of the column. The liquid detention time is also used as a baseline to evaluate potential retardation of target contaminants caused by adsorption. Retardation factors is estimated as the ratio of the time required for 50% breakthrough of BTX in sterile columns to the mean liquid retention time. The dispersion coefficient is estimated by fitting the bromide breakthrough data to an advection/dispersion solution (van Genutchten and Parker, 1984).

5.1.1.4 Analytical Methods

Gas chromatography with mass spectrometry (GC-MS), flame ionization (GC-FID), or electron capture (GC-ECD) is used for measurement of TCE and its degradation products. GC-MS is performed using a Finnigan MAT GCQ mass spectrometer (San Jose, Calif.). A purge and trap extraction system (Tekmar 3000 Purge and Trap, Cincinnati, Ohio) is used for GC-MS analysis of volatiles, as described in EPA Method 8260A. The volatile organic chemicals perchloroethylene, trichloroethylene, dichloroethylenes, vinyl chloride, ethene and ethane are analyzed via these methods.

Reduced iron is analyzed in solution by the ferrozine method or the phenanthroline method (1992 Standard Methods 3500-Fe D), and total dissolved iron is analyzed after 0.2 μm membrane filtration by Perkin Elmer 3300 Atomic Absorption Spectrometer (Norwalk, Conn.) with carbon furnace (Standard Methods 3500-Fe B).

Hexavalent chromium is analyzed calorimetrically using a modified SW-846 Method 7196A. In the absence of interfering amounts of substances such as molybdenum, vanadium, and mercury, Cr(VI) is determined by a red-violet color producing reaction with diphenylcarbizide in acid solution. 95 mL of sample extracted with 3% sodium carbonate (Katz, 1991) is mixed with 2 mL of diphenylcarbazide solution and sulfuric acid is added to bring solution pH to approximately 2. The solution is allowed to stand for 10 min and is then transferred to a 1 cm cuvette for colorimetric measurement at 540 nm. Kazi and Katz (1987) showed that this extraction procedure selectively removes both the soluble and insoluble hexavalent chromium without oxidiz-

ing the trivalent chromium. Digestion of the sample with a sulfuric-nitric acid mixture, and then oxidation with potassium permanganate, converts all chromium species to Cr(VI) (Clesceri et al., 1989). This is then reacted with diphenylcarbide and filtered to determine total chromium. The amount of Cr(III) is determined as the difference between total chromium and Cr(VI). The presence of Cr(III) may be confirmed by electron spin resonance spectroscopy (Lieber et al., 1964).

Total dissolved uranium is measured by radiochemical methods (Standard Methods 7500-U B). Radioisotopes 235, and 238 may be separated by the isotopic method using electrodeposition (Standard Method 7500-U C). These analyses are performed using a Beckman LS6000IC liquid scintillation counter (Fullerton, Calif.).

5.2 Example 2

Fe(0) as an Electron Donor and Energy Source for ISAD

To demonstrate that Fe(0) can serve as the ultimate electron donor and energy source for chemoautotrophic, biological dentrification, a dual-flask apparatus was used (see FIG. **9**, insert). Two 250-ml Erlenmeyer flasks were fused at the top. An opening was made on the side of each flask for sample addition or removal. One flask contained 10 g of Fe(0) and 100 ml of water, and the other flask contained 100 ml of mineral medium with nitrate (40 mg/l as N). The flasks were purged with N_2/CO_2 (80:20, vol./vol.) following inoculation with *P. denitrificans*. This set up permitted the diffusion of cathodic H_2 from the Fe(0)-containing flask through the connecting glass tubing to the seeded flask while avoiding potential microbial inhibition by iron species. Nitrate was removed below detection limits within 5 days in this apparatus with a concomitant increase in microbial concentration as measured by optical density at 600 nm (FIG. **9**). No nitrate removal or microbial growth was observed in control runs, lacking either nitrate, Fe(0) or inoculum, indicating the Fe(0) corrosion and nitrate reduction were coupled via hydrogenotrophic denitrifiers.

Studies were also conducted in batch reactors to investigate nitrate reduction kinetics and to determine biotic and abiotic nitrate removal products in the presence of iron powder (2.02 m^2/g, Aldrich Chemical Co., Milwaukee, Wis.), iron filings (0.14 m^2/g, Fisher Chemical Co., Fairlawn, N.J.), and steel wool (0.0075 m^2/g, Rhodes American, Chicago, Ill.). Serum bottles (250 ml) capped with Miniert® valves were filled with 100 ml of carbonate-buffered, minimal medium with nitrate (50 mg/l as N). Ten g of acid-washed Fe(0) powder, Fe(0) filings, steel wool, or 40 ml H_2 (1 atm) gas were used as electron donors. Some reactors were seeded with hydrogenotrophic denitrifiers, either axenic cultures of *P. denitrificans* ATCC 17741 and *P. denitrificans* (formerly *Thiosphaera pantotropha*), ATCC 35512, or mixed cultures of indigenous aquifer microorganisms enriched with H_2 and nitrate. Control (unseeded) reactors were similarly prepared. The reactors were purged with N_2/CO_2 (80:20, vol./vol.) following inoculation to remove dissolved oxygen and to provide a carbon source of autotrophic growth.

In the absence of bacteria, Fe(0) powder exhibited faster rates for both nitrate removal and H_2 production than Fe(0) filings. This was attributed to the higher specific surface area available for reaction in the Fe(0) powder. In seeded reactors, however the more reactive Fe(0) powder had an inhibitory effect on biological dentrification. This was attributed to the large increase in pH (pH>10) due to Fe(0) corrosion. The inhibitory effect of high pH on dentrification was verified in separate incubations with H_2-fed *P. denitrificans*, which ceased to remove nitrate at pH values greater than 10. Rectors containing Fe(0) filings and bacteria did not exhibit such a large pH increase and completely removed the added nitrate within one month, while abiotic reactors with Fe(0) filings did not. Thus, denitrifiers had a more beneficial effect when combined with the less reactive Fe(0) filings than with the more reactive Fe(0) powder.

Batch studies were also conducted to compare the fate of nitrate when Fe(0) powder, steel wool, or H_2 gas served as electron donors. An axenic culture of *P. denitrificans* was used for inoculation. An acetylene block technique was performed on seeded reactors to quantify biological dentrification (per N_2O accumulation) As in the previous study, faster nitrate removal occurred in reactors containing Fe(0) powder than in reactors containing steel wool or H_2 gas. Incubation times to remove all of the added nitrate (50 mg/l as N) were 8 days for the seeded reactors amended with Fe(0) powder and 12 days for the seeded reactors amended with steel wool or with H_2 gas. Nevertheless, the end products for the reactors containing steel wool more closely resembled the ideal H_2-amended reactors (Table 6), where most of the initial nitrate was biologically denitrified (as shown by N_2O accumulation) rather than reduced to the less favorable product, ammonium, as seen in the reactors containing Fe(0) powder.

TABLE 6

FATE OF NITRATE IN BATCH REACTORS EXPRESSED AS A PERCENTAGE OF THE ADDED 50 MG/L NITRATE (AS N)

Treatment	Unreacted $(N)_3$—N)	Denitrified Biologically (N_2O—N)	Reduced by Fe(0) Abiotically (NH_4^+—N)	Assimilated by Bacteria (Organic N)	Mass Balance Closure
Fe(0) Powder	0	0	98% ± 0.6%	0	98% ± 0.6%
Fe(0) Powder & Bacteria	0	2% ± 0.8%	94% ± 3.0%	2% ± 0.2%	98% ± 1.8%
Steel Wool	68% ± 3.2%	0	30% ± 2.6%	0	98% ± 0.6%
Steel Wool & Bacteria	0	64% ± 10.8%	28% ± 8.0%	2% ± 2.0%	94% ± 1.2%
H_2 Gas & Bacteria	0	93% ± 2.4%	0	1% ± 0.4%	94% ± 0.8%

To investigate the ability of bacteria to sustain nitrate removal in the presence of Fe(0) in a flow-through system, columns packed with steel wool and seeded with mixed cultures of indigenous, aquifer denitrifiers were operated over several months. The columns used were constructed by fusing two 25 mm, threaded-glass connectors (Ace Glass, Vineland, N.J.) together. The columns had an inner diameter of 2.5 cm and were 26.5 cm long. The ends were sealed with

threaded, PFTE stoppers containing fitted-glass filter discs (Ace Glass). Sampling ports were made along the length of the column by making a small opening and fusing a 1.5 dram vial with the bottom cut of onto the column. The sample ports were capped with 13 mm, Teflon®-lined Mininert® valves (Alltech Associates, Inc., Deerfield, Ill.).

Mineral medium containing nitrate (50 mg/l as N) was pumped in an up-flow mode into the columns using a Masterflex® (Barrington, Ill.) 7523-30 peristaltic pump with a 7519-15 pump head. The influent reservoir was a 25-liter Nalgene™ polyethylene carboy (Nalge Co., Rochester, N.Y.). The influent reservoir was continually purged with an N_2/CO_2 (80:20, vol./vol.) gas mix using Masterflex® 6426-16 tubing (3.1 mm inner diameter) with a stone diffuser. The influent tubing was Masterflex® 6402-14 tubing with 1.6 mm inner diameter. The tubing was approximately 2.4 m long, running from the bottom of the influent reservoir to the head of the column. The effluent tubing was Teflon® having an inner diameter of 1.6 mm and a length of about 0.5 m. The end of the effluent tubing was adapted for sampling with a flangeless ferrule and nut arrangement, a 1/4-28 adapter male luer lock fitting. Hamilton (Reno, Nev.) 3-way sampling valves were placed influent and effluent to the columns in order to redirect flow for sample collection, dissolved oxygen measurement, or to stop flow in the system. Preliminary results over 4 months show that this is a sustainable process, and that seeded columns exhibit higher nitrate removal efficiencies (60%) than unseeded controls (40%).

5.3 Example 3

Cathodic H_2 as Electron Donor for $CHCl_3$ Co-Metabolism by a Mixed, Methanogenic Culture

The inventors have exploited the use of elemental iron (Fe^0) for treatment of highly chlorinated organic compounds. While early studies found little microbiological contribution to degradation in laboratory and field tests, recent work has focused exclusively on abiotic processes. In studies conducted with a mixed, methanogenic culture, however, pseudo-first-order rate coefficients for chloroform degradation were at least 3.6 times greater in serum bottle incubations containing 40 mesh iron filings and live cells as compared to incubations containing Fe^0 and killed cells, Fe^0-free incubations with live cells. CF cometabolism and methanogenesis was apparently supported using cathodic hydrogen produced by anaerobic corrosion of the added Fe^0. The use of selective microbial inhibitors showed that H_2-consuming methanogens and not homoacetogens were responsible for CF degradation. The sustainability of the process was established in a 60-day column study using steel wool as support for microbial growth. The observation that cathodically produced H_2 can support reductive dechlorination by anaerobic bacteria has significant practical implications.

5.3.1 Materials and Methods

5.3.1.1 Batch Reactors

Batch studies were conducted in the dark at 20° C. using 25 mL liquid volume in sealed, 38 mL serum bottles. Duplicates were used for all treatments. Bottles were incubated in an inverted position on a circular action shaker table (Lab-Line) at 200 rpm. Initial batch studies examined the effect that amending methanogenic incubations with Fe^0 had on the kinetics of CF transformation. Iron filings (2 g, 40 mesh, specific surface area 0.237 m^2/g, Malinkrodt) were weighed into selected bottles. Bottles were then filled with DI water, sealed with Teflon™-coated rubber septa (West Co., Phoenixville, Pa.), and capped with aluminum crimp caps. Bottles were flushed with N_2/CO_2 gas (80:20, v/v) through the septa to displace the water. A 100 mL glass, gas-tight syringe (Scientific Glass Engineering, Australia) was used to inject bottles with 25 mL of either freshly prepared mineral medium, cell suspension from a stock culture reactor, or autoclaved cell suspension (120° C. for 20 min). The headspace gas exited through a 25-gauge needle. The mineral medium used in these studies was the same as that supplied to the stock culture reactor. Transformation studies were initiated by injecting a volume of CF stock solution through the septum with a 10 μL syringe. CF-free controls containing iron and live cell suspension were used to investigate the inhibition of methanogenesis by CF. Bottles were sampled periodically for CF, DCM, hydrogen, and methane. The liquid-gas mass transfer coefficient, $\kappa_L a$ was determined using the method of Tatara et al. (1993).

A study was conducted to elucidate the possible roles of methanogens and homoacetogens in cathodic hydrogen consumption and CF degradation. Two microbial inhibitors were used for this purpose: bromoethanesulfonic acid (BESA) and vancomycin. BESA is a specific methanogenic inhibitor (Sparling and Daniels, 1987). Vancomycin is an antibiotic that inhibits eubacterial cell wall formation (Bock and Kandler, 1985). Incubations containing 2 g of iron filings were amended with neither, one, or both of the inhibitors at concentrations of 50 mM BESA (Zinder et al., 1984; Aguilar et al., 1995) or 100 mg/L vancomycin (DiStefano et al., 1992; Aguilar et al., 1995; Freedman and Gossett, 1989; Perkins et al., 1994). Fe^0-free incubations with a N_2/CO_2 headspace received 2 mL of H_2/CO_2 gas and neither, one, or both of the inhibitors at the same concentrations. The bottles were sampled daily for hydrogen and methane. When hydrogen reached low levels in some of the H_2-amended incubations, the headspace of all of the H_2-additional 2 mL of H_2/CO_2 gas was injected into the serum bottles. This cycle was repeated once more, and this time a volume of CF-saturated stock was also injected into the bottles.

5.3.1.2 Column Reactors

A study employing column reactors was conducted over a 60-d period to determine if the CF degradation activity observed with batch reactors was sustainable. Three glass chromatography columns (2 cm i.d.×20 cm) were used to study the transformation of CF under continuous-flow conditions. Steel wool (0.0075 m^2/g, Medium 1, Rhodes/American, Chicago, Ill.) was used as an iron source and physical support for the attachment of bacteria. The chemical composition of the steel wool, as reported by the manufacturer, was (in %) Fe (52), Si (30), C (16), Mn (1.25), P (0.7), and S (0.05). Two columns were packed end-to-end with 5.5 g sections of an unrolled steel wool pad. The third column was used as a sterile (autoclaved) control to evaluate volatilization losses and was filled with 5 mm-diameter glass beads. One steel wool-filled column was seeded with two 100 mL aliquots of cell suspension from the stock culture reactor using a 100-mL glass, gas-tight syringe. The microorganisms were allowed to colonize the steel wool for 2 days with no flow to the column. The columns were then operated for 2 w with CF-free influent, using the same medium that was supplied to the stock culture reactor, but without the addition of acetic acid. The medium was buffered by adding 1 mL of 1 N HCl to 200 mL of fresh medium and then adjusting the pH to 6.7 with $NaHCO_3$. After 2 w, CF was

added to the feed solution from the CF stock solution using a 10 μL syringe. Influent was pumped into the columns through Teflon™ tubing from 25 mL glass, gas-tight syringes (Hamilton) with a syringe pump (Harvard Apparatus). The columns were fed upflow at a volumetric flow rate of 7.9 mL/d. The porosity of the steel wool- and glass bead-filled columns was 0.90 and 0.30, respectively, resulting in superficial velocities of 2.8 and 8.4 cm/d and hydraulic retention times of 7.2 and 2.4 d, respectively. Effluent samples were taken with disposable syringes attached to Teflon™ tubing, which extended to the top of the steel wool or glass beads.

5.3.1.3 Chemicals and Stock Solutions

CF (high-performance liquid chromatography (HPLC) grade) and DCM (certified American Chemical Society (ACS) grade) were purchased from Fisher Scientific (Pittsburgh, Pa.). Stock aqueous solutions of CF and DCM were prepared by adding about 5 mL of each chemical to 25 mL of autoclaved, distilled deionized water in 43 mL glass serum bottles sealed with Teflon™-lined rubber septa and aluminum crimp caps. Other chemicals used included acetic acid (glacial, Malinkrodt), methane gas (100%, Scott Specialty Gases), 2-bromoethanesulfonic acid (98%, Aldrich Chemical Company, Milwaukee, Wis.), and vancomycin (Sigma Chemical Company, St. Louis, Mo.).

5.3.1.3 Stock Culture Reactor

The source of organisms was a magnetically stirred, 9.5 L glass reactor containing an acetate-enriched methanogenic cell suspension volume of 8 L. The reactor was maintained at 20° C. with a 40-d hydraulic retention time, such that 200 mL of cell suspension was removed daily and replaced with fresh medium. The medium recipe has been listed previously (Hughes and Parkin, 1996). The medium was buffered with $NaHCO_3$ as needed to maintain a reactor pH of 6.9±0.1. The volatile suspended solids concentration of the reactor averaged 245±20 mg/L (n=5) at the time the studies were conducted.

5.3.1.4 Analytical Methods

CF, DCM, H_2, and CH_4 were determined by gas chromatography (GC) using headspace analysis. Headspace samples were withdrawn using a locking, gas-tight syringe (Precision Sampling Corp., Baton Rouge, La.) equipped with a 22-gauge side-port needle and then injected into a GC. The headspace of 38 mL batch reactors was directly sampled by this method. For the column reactors, a 1 mL aqueous sample was taken from an effluent sample port with a 3 mL disposable plastic syringe and, using a 25-gauge needle, injected into a 5 mL glass vial sealed with a Teflon™-coated, rubber septum and screw cap. The headspace of this bottle was then injected into a GC.

CF was analyzed using an HP 5890 Series II GC equipped with an electron capture detector and a DB-5 capillary column (J and W Scientific, Folsom, Calif.). DCM and CH_4 were analyzed on a HP 5890 Series II GC equipped with a flame ionization detector and a DB-WAX capillary column (J and W Scientific). H_2 was analyzed on a HP 5890 Series II GC equipped with a thermal conductivity detector using a Hayesep Q packed column (Alltech Associates). For the 38 mL batch reactors, sample sizes for CF, DCM, H_2, and CH_4 were 100, 500, 100, and 100 μL, respectively, with corresponding detection limits of 1.7, 15.3, 188, and 9.3 nmol/bottle, respectively. For the column reactors, a 500 μL sample size was used for CF, DCM, and CH_4, and detection limits were 0.07, 0.78, and 2 μM, respectively.

Acetate concentrations were determined by HPLC analysis using a PRP-X300 column (Hamilton), Gilson Model 306 pump, and Model 805 manometric module (pulse dampener). Peak areas were integrated using Gilson 712 Controller Software version 1.2. The detection limit was approximately 0.08 mM.

Biomass was measured as volatile suspended solids using Method 2540 E in *Standard Methods* (APHA, 1985). The pH was measured with a pH meter (Beckman Model F 72) and combination electrode (Fisher Scientific, Pittsburgh, Pa.).

5.3.2 Results

5.3.2.1 Batch Reactors

CF was transformed most rapidly in the incubations containing live cells and Fe^0 (FIG. **10**A). DCM was detected only in the treatments containing cells and Fe^0 (FIG. **10**B). The H_2 concentration in the incubations containing live cells and Fe^0 differed sharply depending on the presence of CF. H_2 behavior in the incubation containing live cells, Fe^0, and CF was similar to H_2 behavior in the treatment containing mineral medium and iron until the CF concentration in the live cell-Fe^0—CF treatment reached low levels, whereas H_2 in the CF-free incubation containing live cells and Fe^0 remained at low levels throughout (FIG. **10**C). Likewise, CF affected methane production in the live cell-Fe^0 treatments; low levels of methane were measured in the live cell-Fe^0 incubation containing CF until the CF concentration reached low levels, while methane was steadily produced at a faster rate in the CF-free live cell-Fe^0 incubation (FIG. **10**D).

CF was rapidly transformed and DCM was produced when Fe^0-free incubations containing live cells were amended with 2 mL of H_2/CO_2 (80:20, v/v) gas (FIG. **11**). The slow disappearance of CF in the H_2-free control containing live cells indicated that an external electron donor was required for rapid CF transformation. Furthermore, adding H_2 to incubations containing autoclaved cell suspension had little effect on CF transformation, indicating that CF transformation using H_2 as an electron donor was enzymatic.

FIG. **12**A and FIG. **12**B show the impact of the inhibitors on cathodic hydrogen and methane levels in the incubations containing cells and iron. The behavior of the incubations was controlled by the methanogenic inhibitor BESA: bottles containing BESA alone or BESA and vancomycin responded similarly, while bottles containing vancomycin alone or no inhibitor behaved similarly. Hydrogen levels were similar in all bottles for the first 2 days. After this, the H_2 concentration in the BESA-amended incubation sets continued to increase, while the concentration of H_2 in the vancomycin or inhibitor-free incubations decreased, eventually becoming nondetectable (FIG. **12**A). Similarly, methanogenesis was inhibited in both incubation sets containing BESA as compared to the inhibitor-free incubations or the incubations containing vancomycin only (FIG. **12**B).

FIG. **13**A, FIG. **13**B, and FIG. **13**C show the impact of the inhibitors on hydrogen consumption, methane production, and CF transformation in incubations supplied with H_2. In general, the behavior of the incubations was controlled by BESA, and vancomycin alone had no effect. During the first 2 days, neither of the inhibitors appeared to affect H_2 consumption. After this, however, the rate of H_2 consumption in the incubations containing vancomycin or no inhibitor remained steady, while the rate of H_2 consumption in the incubations amended with BESA alone or BESA and van-

comycin decreased (FIG. **13**A). The relatively constant H_2 concentration in the control reactor confirmed that the decrease in the H_2 levels in the live cell incubations was not due to volatile losses. Acetate was not detected (<0.08 mM) in liquid samples taken from the incubations during the first 3 days. Methane production in the incubations containing BESA alone or BESA and vancomycin was severely inhibited in comparison to methane production in the vancomycin- or inhibitor-free incubations (FIG. **13**B). When bottles were resupplied with H_2, vancomycin had no effect on either H_2 consumption or methane production, while BESA severely inhibited both (FIG. **13**A and FIG. **13**B). Bottles were resupplied with H_2 on the ninth day and spiked with CF. Vancomycin alone had no impact on CF transformation (FIG. **13**C). Moreover, the rate of CF transformation in incubations amended with BESA alone or BESA and vancomycin was similar and lower than in BESA-free incubations. Hydrogen consumption and methane production were inhibited in the presence of CF.

5.3.2.2 Continuous-Flow Column Studies

The CF concentration in the effluent from the abiotic, steel wool column, averaging 0.41±0.17 μM, was consistently greater than that from the methanogenic, steel wool column, which averaged 0.00±0.02 μM CF (FIG. **14**A). In fact, on only one occasion was CF detectable in the methanogenic column effluent. A small peak on chromatograms was visible at the DCM elution time (2.3 min) in samples from these two columns on several sampling events; however, this peak was not integrated. The effluent from the glass bead control column, 1.29±0.85 μM CF, was not statistically different (at the 95% level) from the influent CF concentration, 1.61±0.49 μM CF. This indicated that the loss of CF addition was initiated and increased 1 order of magnitude during the next 36 d to 7.43 mg of CH_4/L on d 61 (FIG. **14**B). The pH of the effluent from the methanogenic, steel wool column and the abiotic, steel wool column was 8.3 and 8.7, respectively.

5.3.3 Discussion

When the data shown in FIG. **4**A was plotted as $\ln(M/M_0)$ versus t (where M is the total mass of CF (nmol of CF) in at bottle at time t, and M_0 is the initial mass of CF (nmol of CF)), linear plots were produced, indicating that CF transformation rates followed first-order kinetics. Slopes of these plots, i.e., pseudo-first-order rate coefficients, $\kappa(d^{-1})$, were then compared. The CF transformation rate coefficient for the treatment containing Fe^0 and live cells, 0.72 d^{-1} (r^2= 0.96), was greater than the value of κ for the treatment containing live cells only, 0.10 d^{-1} (r^2=0.81); killed cells and Fe^0, 0.20 d^{-1} (r^2=0.99); and mineral medium and Fe^0, 0.08 d^{-1} (r^2=0.63). The large value of the gas-liquid mass transfer coefficient, $\kappa_L a$, 95 d^{-1}, ensured that CF transformation kinetics were not limited by the rate of mass transfer between the liquid and gas phase.

Interestingly, combining Fe^0 and live cells was synergistic with respect to CF degradation; the rate coefficient for the combined Fe^0 and live cell treatment was 2.4 times the sum of the rate coefficients for the separate treatments containing Fe^0 or live cells. Hence, abiotic and microbial CF degradation processes were not independent when Fe^0 and cells were combined in a single reactor. This synergism apparently resulted from the production of hydrogen as a result of the oxidation of Fe^0 (Reardon, 1996), which was then used as a primary substrate for CF cometabolism. The detection of hydrogen in the incubations to which Fe^0 was added (FIG. **4**C) and the stimulation of CF degradation when hydrogen was added directly support these conclusions (FIG. **5**).

Reductive dechlorination is an electron-consuming process, the stimulation of which by the addition of an exogenous electron donor is common (Bagley and Gossett, 1995; Freedman and Gossett, 1989; Fathepure and Boyd, 1988). Although H_2 stimulated CF degradation, CF inhibited hydrogen consumption and methane production (FIG. **4**C and FIG. **4**D), which is a common effect of CF on methanogenic systems (Hughes and Parkin, 1996; Bauchop, 1967; Thiel, 1969; Swanwick and Foulkes, 1971; Prins et al., 1972; Yang and Speece, 1986; Hickey et al., 1987). It should be noted that, while the consumption of H_2 in incubations containing CF is not noticeable (FIG. **10**C), only 1 nmol of H_2 is required for the reduction of 1 nmol of $CHCl_3$ to CH_2Cl_2, according to

$$CHCl_3+H_2 \rightarrow CH_2Cl_2+H^++Cl^- \quad \text{(Equation 17)}$$

Consequently, during the first 3 d, for example, a negligible amount (about 0.1%) of the H_2 produced (at least 60 μmol of H_2) would have been required for the degradation of about 80 nmol of CF.

The studies conducted with microbial inhibitors support the hypothesis that methanogenic bacteria were responsible for cathodic hydrogen consumption and CF dechlorination using cathodic hydrogen as electron donor. Vancomycin, shown by Murray and Zinder (1984) to inhibit eubacteria at the concentration used in this study, 100 mg/L, had little or no impact on cathodic hydrogen consumption or methanogenesis when added alone to bottles containing iron (FIG. **12**A and FIG. **12**B). In contrast, BESA, a methanogenic inhibitor, inhibited both of these processes to a similar degree regardless if added alone or with vancomycin (FIG. **12**A and FIG. **12**B). Similar results were seen in treatments to which hydrogen was added directly: vancomycin had no impact on hydrogen consumption or methanogenesis when added alone, while both of these processes were inhibited in bottles that contained BESA alone or BESA and vancomycin (FIG. **13**A, FIG. **13**B). Utilization of hydrogen by methanogens for metabolic purposes may account for the decrease in hydrogen concentration in the BESA-inhibited incubations in the early stage of this study. Acetate, a product of homoacetogens, was not detected. Furthermore, vancomycin by itself had no impact on CF degradation as compared to an inhibitor-free control (FIG. **13**C). In contrast, CF degradation was inhibited to the same degree in bottles containing BESA alone or BESA and vancomycin, compared to an inhibitor-free control (FIG. **7**C). The slow rate of CF degradation in the BESA-inhibited bottles supports the hypothesis that methanogens were responsible for CF degradation. BESA is a structural analogue of coenzyme M (2-mercaptoethane sulfonic acid) (Sparling and Daniels, 1987), which is unique to methanogens (Balch and Wolfe, 1979). The prosthetic group of methyl reductase is coenzyme F_{430} (Ellefson et al., 1982), a nickel porphynoid that facilitates the reduction of CF and other chlorinated aliphatics in abiotic studies when a bulk reducing agent such as titanium (III) citrate or dithiothreitol is provided (Krone et al., 1989a; Gantzer and Wackett, 1991). Methanogens contain other metallocoenzymes, such as cobalt corronoids, that are not directly affected by BESA and that have also been shown to reduce CF and other chlorinated aliphatics (Gantzer and Wackett, 1991; Krone et al., 1989b; 1991; Assaf-Anid et al., 1994; Chiu and Reinhard, 1995; 1996; Stromeyer et al., 1992; Lewis et al., 1996). Hence, although BESA may completely inhibit methanogenesis and F_{430}-mediated CF reduction, BESA may not stop the degradation of CF by pure or mixed methanogenic cultures.

The extent of CF removal in the microbial steel wool column was consistently greater than in the abiotic column during the 60-d experimental period. This illustrates that CF cometabolism by anaerobic bacteria using cathodic hydrogen as the sole electron donor is sustainable, a finding that was uncertain based on the batch studies where methanogenesis was strongly inhibited in the presence of CF (FIG. **10**A, FIG. **10**B, FIG. **10**C, and FIG. **10**D). The increase in the concentration of methane in the effluent from the microbial steel wool column no doubt reflects the growth of hydrogenotrophic methanogens within the column as well as acclimation to CF. Based on the presumed increase in methanogenic biomass during the study, it is reasonable to assume that higher influent CF concentrations than those studied here could be reduced to nondetectable levels in the effluent.

This example illustrates the utility of microbial metallic iron treatment systems as an advantage over abiotic zero-valent iron schemes. In a field-scale application of this technology, cells grown in a fermenter or sludge from an anaerobic digester could be injected into a permeable, iron-containing, reactive barrier similar to the reactor originally proposed by Blowes et al. (1995). For some compounds, combined microbial abiotic systems may accelerate the rate of transformation and extent of mineralization; dichloromethane, a CF dechlorination product, does not undergo measurable abiotic transformation by iron (Matheson and Tratnyek, 1994) but can be utilized as a growth substrate by acclimated anaerobes (Freedman and Gossett, 1991; Stromeyer et al., 1991; Braus-Stromeyer et al., 1993; Maegli et al., 1995). Conversely, anaerobic bacteria reduct TCE to ethene or ethane via dichloroethylene and vinyl chloride (Freedman and Gossett, 1989; Wild et al., 1995), while abiotic iron systems reduce TCE to ethene and ethane without the production of these intermediates (Orth and Gillham, 1995).

6.0 REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference:

U.S. Pat. No. 4,554,101, issued Nov. 19, 1985.

Adelman, Hayflick, Vasser, Seeburg, "In vitro deletional mutagenesis for bacterial production of the 20,000-dalton form of human pituitary growth hormone," *DNA,* 2(3): 183–193, 1983.

Aguilar, Casas, Lema, "Degradation of volatile fatty acids by differently enriched methanogenic cultues: Kinetics and inhibition," *Water Res.,* 29(2):505–509, 1995.

Alvarez and Vogel, "Substrate interactions of Benzene, Toluene, and para-Xylene during microbial degradation by pure cultures and mixed culture aquifer slurries," *Appl. Environ. Microbiol.,* 57(10):2981–2985, 1991.

APHA, *Standard Methods for the Examination of Water and Wastewater,* 16th ed., American Public Health Association, Washington, D.C., 1985.

Assaf-Anid, Hayes, Vogel, "Reductive dechlorination of carbon tetrachloride by cobalmin (II) in the presence of dithiothreitol: Mechanistic study, effect of redox potential and pH," *Environ. Sci. Technol.,* 28(2):246–252, 1994.

Bagley and Gossett, "Chloroform degradation in methanogenic enrichment cultures and by *Methanosarcina barkeri* 227," *Appl. Environ. Microbiol.,* 61:3195–3201, 1995.

Balch and Wolfe, "Specificity and biological distribution of coenzyme M (2-mercaptoethanesulfonic acid)," *J. Bacteriol.,* 137(1):256–263, 1979.

Bauchop, "Inhibition of rumen methanogenesis by methane analogues," *J. Bacteriol.,* 94(1):171–175, 1967.

Belay and Daniels, "Elemental metals as electron sources for biological methane formation from carbon dioxide," *Antonie van Leeuvenhoek,* 57(1):1–8, 1990.

Blowes, Ptacek, Cherry, Gillham, Robertson, In: *Geoenvironment 2000: Characterization, Containment, Remediation, and Performance in Environmental Geotechnics,* Yalcin B A, Daniel D E, eds., Geotechnical Special Publication No. 46, American Society of Civil Engineers, New York, 2:1608–1621, 1995.

Bock and Kandler, In: *The Bacteria: A Treatise on Structure and Function, Volume VIII, Archaebacteria*, Woese C R, Wolfe R S, eds, Academic Press, New York, pp. 525–544, 1985.

Bolivar, Rodriguez, Greene, Betlach, Heynecker, Boyer, "Construction and characterization of new cloning vehicles. II. A multipurpose cloning system," *Gene,* 2(2): 95–113, 1977.

Bouwer and McCarty, "Transformations of 1- and 2-carbon halogenated aliphatic organic compounds under methanogenic conditions," *Appl. Environ. Microbiol.,* 45:1286–1294, 1983.

Bouwer, Rittmann, McCarty, "Anaerobic degradation of halogenated 1 carbon and 2 carbon organic compounds," *Environ. Sci. Technol.,* 15(5):596–599, 1981.

Braus-Stromeyer, Hermann, Cook, Leisinger, "Dichloromethane as the sole carbon source for an acetogenic mixed culture and isolation of a fermentative, dichloromethane-degrading bacterium," *Appl. Environ. Microbiol.,* 59(11):3790–3797, 1993.

Brock et al., "Biology of Microorganisms" 7th Edition, Prentice Hall, Inc., Englewood Cliffs, N.J., 1994.

Capecchi, M. R., "High efficiency transformation by direct microinjection of DNA into cultured mammalian cells," *Cell,* 22(2):479–488, 1980.

Chang, Nunberg, Kaufman, Erlich, Schimke, Cohen, "Phenotypic expression in *E. coli* of a DNA sequence coding for mouse dihydrofolate reductase," *Nature,* 275:617–624, 1978.

Chiu and Reinhard, "Metallocoenzyme-mediated reductive transformation of carbon tetrachloride in titanium (III) citrate aqueous solution," *Environ. Sci. Technol.,* 29(3): 595–603, 1995.

Chiu and Reinhard, *Environ. Sci. Technol.,* 30:1882–1889, 1996.

Chou and Fasman, "Conformational Parameters for Amino Acides in Helical, β-Sheet, and Random Coil Regions Calculated from Proteins," *Biochemistry,* 13(2):211–222, 1974b.

Chou and Fasman, "Empirical Predictions of Protein Conformation," *Ann. Rev. Biochem.,* 47:251–276, 1978b.

Chou and Fasman, "Prediction of β-Turns," *Biophys. J.,* 26:367–384, 1979.

Chou and Fasman, "Prediction of Protein Conformation," *Biochemistry,* 13(2):222–245, 1974a.

Chou and Fasman, "Prediction of the Secondary Structure of Proteins from Their Amino Acid Sequence," *Adv. Enzymol. Relat. Areas Mol. Biol.,* 47:45–148, 1978a.

Clesceri et al., *Standard Methods for the Examination of Water and Wastewater,* APHA-AWWA-WPCF, 17th Edition, 1989.

Criddle et al., "Transformation of carbon tetrachloride by Pseudomonas sp. strain KC under denitrifying conditions," *Appl. Environ. Microbiol.,* 56:3240–3246, 1990.

Daniels et al., "Bacterial methanogenesis and growth from CO_2 with elemental iron as the sole source of electrons," *Science,* 237:509–511, 1987.

DiStefano, Gossett, Zinder, "Hydrogen as an electron donor for dechlorination of tetrachloroethene by an anaerobic mixed culture," *Appl. Environ. Microbial.,* 58(11): 3622–3629, 1992.

Doong and Wu, "Reductive dechlorination of chlorinated hydrocarbons in aqueous solutions containing ferrous and sulfide ions," *Chemosphere,* 24:1063–1075, 1992.

Egli, Scholtz, Cook, Leisinger, "Anaerobic dechlorination of tetrachloromethane and 1,2-dichloroethane to degradable products by pure cultures of Desulfobacterium sp. and Methanobacterium sp." *FEMS Microbiol. Lett.,* 43:257–261, 1987.

Egli, Stromeyer, Cook, Leisinger, "Transformation of tetrachloromethane and trichloromethane to carbon dioxide by anaerobic bacteria is a non-enzymatic process," *FEMS Microbiol. Lett.,* 68(1–2):207–212, 1990.

Egli, Tschan, Scholtz, Cook, Leisinger, "Transformation of tetrachloromethane to dichloromethane and carbon dioxide by *Acetobacterium woodii,*" *Appl. Environ. Microbiol.,* 54(11):2819–2824, 1988.

Eglitis and Anderson, "Retroviral vectors for introduction of genes into mammalian cells," *Biotechniques,* 6(7): 608–614, 1988.

Eglitis, Kantoff, Kohn, Karson, Moen, Lothrop, Blaese, Anderson, "Retroviral-mediated gene transfer into hemopoietic cells," *Avd. Exp. Med. Biol.,* 241:19–27, 1988.

Eichenlaub, "Mutants of the mini-F plasmid pML31 thermosensitive in replication," *J. Bacteriol.,* 138(2): 559–566, 1979.

Ellefson, Whitman, Wolfe, "Nickel-containing factor F_{430}: chromophore of the methylreductase of Methanobacterium," *Proc. Natl. Acad. Sci. USA,* 79(12): 3707–3710, 1982.

Fathepure and Boyd, "Dependence of tetrachloroethylene dechlorination on methanogenic substrate consumption by Methanosarcina sp. strain DCM," *Appl. Environ. Microbiol.,* 54(12):2976–2980, 1988.

Fathepure, Tiedje, Boyd, "Reductive dechlorination of hexachlorobenzene to tri- and dichlorobenzenes in anaerobic sewage sludge," *Appl. Environ. Microbiol.,* 54(2):327–330, 1988.

Fathepure and Tiedje, "Reductive dechlorination of tetrachloroethylene by a chlorobenzoate-enriched biofilm reactor," *Environ. Sci. Technol.,* 28:746–752, 1994.

Fiers, Contreras, Haegemann, Rogiers, Van de Voorde, Van Heuverswyn, Van Herreweghe, Volckaert, Ysebaert, "Complete nucleotide sequence of SV40 DNA," *Nature,* 273(5658):113–120, 1978.

Freedman and Gossett, "Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions," *Appl. Environ. Microbiol.,* 55(9):2144–2151, 1989.

Freedman and Gossett, "Biodegradation of dichloromethane and its utilization as a growth substate under methanogenic conditions," *Appl. Environ. Microbiol.,* 57(10): 2847–2857, 1991.

Fromm, Taylor, and Walbot, "Expression of genes transferred into monocot and dicot plant cells by electroporation," *Proc. Natl. Acad. Sci. USA,* 82(17): 5824–5828, 1985.

Galli and McCarty, "Biotransformation of 1,1,1-trichloroethane, trichloromethane, and tetrachloromethane by a Clostridium sp.," *Appl. Environ. Microbiol.,* 55:837–844, 1989.

Gantzer and Wackett, "Reductive dechlorination catalyzed by bacterial transition-metal coenzymes," *Environ. Sci. Technol.,* 25(4):715–722, 1991.

Gefter, Margulies, Scharff, "A simple method for polyethylene glycol-promoted hybridization of mouse myeloma cells," *Somat. Cell Genet.,* 3(2):231–236, 1977.

Gillham and O'Hannesin, "Enhanced degradation of halogenated aliphatics by zero-valent iron," *Groundwater,* 32(6):958–967, 1994.

Goeddel, Heyneker, Hozumi, Arentzen, Itakura, Yansura, Ross, Miozzari, Crea, Seeburg, "Direct expression in *Escherichia coli* of a DNA sequence for human growth hormone," *Nature,* 281(5732):544–548, 1979.

Goeddel, Shepard, Yelverton, Leung, Crea, Sloma, Pestka, "Synthesis of human fibroblast interferon by *E. coli,*" *Nucl. Acids Res.,* 8(18):4057–4074, 1980.

Goel, Kumar, Payne, and Dube, "Plant cell biodegradation of a xenobiotic nitrate ester, nitroglycerin," *Nature Biotechnology,* 15:174–177, 1977.

Gorby and Lovely, "Enzymatic uranium precipitation," *Environ. Sci. Technol.,* 30:205–207, 1992.

Gossett, "Anaerobic degradation of C1 and C2 chlorinated hydrocarbons," ESL-TR-85-38, Air Force Engineering and Services Center, Tyndall Air Force Base, Fla., 1985.

Gould, "The kinetics of hexavalent chromium reduction by metallic iron," *Water Res.,* 26:871–877, 1992.

Graham and van der Eb, "Transformation of rat cells by DNA of human adenovirus 5," *Virol.,* 54(2):536–539, 1973.

Green, Issemann, Sheer, "A versatile in vivo and in vitro eukaryotic expression vector for protein engineering," *Nucl. Acids Res.,* 16(1):369, 1988.

Guan et al., "Sorption rates for the uptake of Cr^{+6} by a consortia of denitrifying bacteria," *Biotechnol. Lett.,* 15(7):733–736, 1993.

Hamilton, "Sulphate-reducing bacteria and anaerobic corrosion," *Annu. Rev. Microbiol.,* 39:195–217, 1985.

Hansen, Koch, Borggaard, and Sorensen, "Abiotic nitrate reduction to ammonium: Key role of green rust," *Envir. Sci. Technol.,* 30(6):2053–2056, 1996.

Helland et al., "Reductive dechlorination of carbon tetrachloride with elemental iron," *J. Hazard Mater.,* 41:205–216, 1995.

Hess et al., *J. Adv. Enzyme Reg.,* 7:149, 1968.

Hickey, Vanderwielen, Switzenbaum, "The effects of organic toxicants on methane production and hydrogen gas levels during the anaerobic digestion of waste activated sludge," *Water Res.,* 21(11):1417–1428, 1987.

Hitzeman, Clarke, Carbon, "Isolation and characterization of the yeast 3-phosphoglycerokinase gene (PGK) by an immunological screening technique," *J. Biol. Chem.,* 255 (24):12073–12080, 1980.

Holland and Holland, "Isolation and identification of yeast messenger ribonucleic acids coding for enolase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoglycerate kinase," *Biochemistry,* 17(23):4900–4907, 1978.

Holliger et al., "A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethylene," *Appl. Environ. Microbiol.,* 59:2991–2997, 1993.

Holliger, "The anaerobic microbiology and biotreatment of chlorinated ethenes," *Curr. Opin. Biotechnol.,* 6:347–351, 1995.

Hughes and Parkin, "Concentration effects on chlorinated aliphatic transformation kinetics," *J. Environ. Eng.,* 122:92–98, 1996.

Ishibashi, Cervantes, Silver, "Chromium reduction in *Pseudomonas putida,*" *Appl. Environ. Microbiol.,* 56(7): 2268–2270, 1990.

Itakura, Hirose, Crea, Riggs, Heyneker, Bolivar, Boyer, "Expression in *Escherichia coli* of a chemically synthe-

sized gene for the hormone somatostatin," *Science,* 198 (4321):1056–1063, 1977.

Johnson et al., "Kinetics of halogenated organic compound degradation by iron metal." *Environ. Sci. Technol.,* 30:2634–2640, 1996.

Jones, "Proteinase mutants of *saccharomyces-cerevisiae," Genetics,* 85(1):23–33, 1977.

Kaplan et al., "Formation of a barrier to groundwater contaminants by injection of zero-valent iron colloids: suspension properties," *Proc. In Situ Remediation: Scientific Basis for Current and Future Technologies Symposium. Thirty-third Hanford Symposium on Health and the Environment;* Nov. 7–11, 1994.

Katz, "The analytical biochemistry of chromium," *Environmental Health Perspectives,* 92I.13–16, 1991.

Kazi and Katz, "The biocycling of some micronutrients and some toxic elements from sewage sludge used as agricultural fertilizers," Second International Conference on Health Disease, Karachi, 1987.

Khudenko, "Mathematical models of cementation processes," *ASCE J. Environ. Engrg.,* 113:681–702, 1987.

Kingsman, Clarke, Mortimer, Carbon, "Replication in *Saccharomyces cerevisiae* of plasmid pBR313 carrying DNA from the yeast trp1 region," *Gene,* 7(2):141–152, 1979.

Koch and Hansen, "Reduction of nitrate to ammonium by sulphate green rust," *Advances in GeoEcology,* 30:373–393, 1997.

Kohler and Milstein, "Continuous cultures of fused cells secreting antibody of predefined specificity," *Nature,* 256 (5517):495–497, 1975.

Kohler and Milstein, "Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion," *Eur. J. Immunol.,* 6(7):511–519, 1976.

Kriegman-King and Reinhard, "Abiotic transformation of carbon tetrachloride at mineral surfaces," U.S. EPA, EPA/600/SR-94/018, March, 1994.

Krone, Laufer, Thauer, Hogenkamp, "Coenzyme F_{430} as a possible catalyst for the reductive dechlorination of chlorinated C_1 hydrocarbons in methanogenic bacteria," *Biochemistry,* 28(26):10061–10065, 1989a.

Krone, Thauer, Hogenkamp, "Reductive dehalogenation of chlorinated C-1-hydrocarbons mediated by corrinoids," *Biochemistry* 28(11):4908–4914, 1989b.

Krone, Thauer, Hogenkamp, Steinbach, "Reductive formation of carbon monoxide from carbon tetrachloride and freons 11, 12 and 13 catalyzed by corrinoids," *Biochemistry,* 30(10):2713–2719, 1991.

Lewis, Morra, Brown, "Comparative product analysis of carbon tetrachloride dehalogenation catalyzed by cobalt corrins in the presence of thiols or titanium (III) reducing agents," *Environ. Sci. Technol.,* 30(1):292–300, 1996.

Lewis, David G., "Factors influencing the stability and properties of green rust," *Advances in GeoEcology,* 30:345–372, 1997.

Lieber et al., "Cadmium and hexavalent chromium in Nassau County groundwater," *Water Works Assoc.,* 56:739–747, 1964.

Longmire et al., "Hydrogeochemical Interactions and Evolution of Acidic Solutions in Soil," In: *Chemical Modeling of Aqueous Systems II,* Melchior and Bassett, (Eds.), ACS Symposium Series 416, American Chemical Society, Washington, D.C., pp. 154–168, 1990.

Lorowitz, Nagle, Tanner, "Anaerobic oxidation of elemental metals coupled to methanogenesis by *methanobacterium-thermoautotrophicum," Environ. Sci. Technol.,* 26(8): 1606–1610, 1992.

Lovely and Phillips, "Bioremediation of uranium contamination with enzymatic uranium reduction," *Environ. Sci. Technol.,* 26:2228–2234, 1992b.

Lovely and Phillips, "Reduction of Uranium by *Desulfovibrio desulfuricans," Appl. Environ. Microbiol.,* 58:850–856, 1992a.

Maegli, Rainey, Leisinger, "Acetogenesis from dichloromethane by a two-component mixed culture comprising a novel bacterium," *Appl. Environ. Microbiol.,* 61(8): 2943–2949, 1995.

Maloy et al., "Microbial Genetics" 2nd Edition. Jones and Barlett Publishers, Boston, Mass., 1994.

Maloy, In: *Experimental Techniques in Bacterial Genetics,* Jones and Bartlett Publishers, Boston, Mass., 1990.

Maniatis et al., "Molecular Cloning: a Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982.

Matheson and Tratnyek, "Reductive dehalogenation of chlorinated methanes by iron metal," *Environ. Sci. Technol.,* 28:2045–2053, 1994.

Matheson, Leah J., "Abiotic and biotic processes affecting the dechlorination of chlorinated solvents by zero-valent iron," *Abstracts of Papers,* 20th ACS National Meeting, American Chemical Society, Mar. 13–17, 1994, Abstract No. 135.

Metcalf and Eddy, Inc., *Wastewater engineering: treatment, disposal, and reuse,* 3rd ed., McGraw-Hill, New York, 1991.

Mikesell and Boyd, "Dechlorination of chloroform by Methanosarcina strains," *Appl. Environ. Microbiol.,* 56:1198–1201, 1990.

Morrison, Spangler, and Tripathi, "Coupled Reaction/Transport Modeling of a Chemoical Barrier for Controlling Uranium (VI) contamination in Groundwater:. *J. Contaminant Hydrology,* 17:343–363, 1995.

MSE Technology Applications. "Analysis of Technologies for the Emplacement and Performance Assessment of Subsurface Reactive Barriers for DNAPL Containment". Report for U.S. Department of Energy (TTP# PE1-6-PL-341) under Contract No. DE-AC22-88ID12735, 1996.

Murray and Zinder, "Nitrogen fixation by a methanogenic archaebacterium," *Nature,* 312(5991):284–286, 1984.

Myneni, Tokunaga, and Brown, "Abiotic selenium redox transformations in the presence of Fe(II,III) oxides," *Science,* 278:1106–1109, 1997.

National Research Council, "Alternatives for groundwater cleanup," *Report of the National Academy of Science Committee on Groundwater Cleanup Alternatives,* National Academy Press, Washington, D.C., 1994.

O'Hannesin and Gillham, "A permeable reaction wall for in situ degradation of halogenated organic compounds," *Proc. 5th Canadian Geotechnical Society Conference,* Toronto, Ontario, Canada, 1992.

Olowe, Benbouzid-Rollet, Génin, Prieur, Confente and Resiak, "La présence simultanée de rouille verte 2 et de bactéries sulfato-réductrices en corrosion perforante de palplanches en zone portuaire," *C.R. Acad. Sci. Paris,* t. 314, Série II, pl. 1157j-1163, 1992.

Ormerod, "Science and secrecy," *Nature,* 350(6319): 551–552, 1991.

Orth and Giliham, "Dechlorination of trichloroethylene in aqueous solution using Fe(0)," *Environ. Sci. Technol.,* 30:66–71, 1996.

Perkins, Koimisar, Puhakka, Ferguson, "Effects of electron donors and inhibitors on reductive dechlorination of 2,4,6-trichlorophenol," *Water Res.,* 28(10):2101–2107, 1994.

Powell et al., "Coupled iron corrosion and chromate reduction: mechanisms for subsurface remediation," *Environ. Sci. Technol.,* 29:1913–1922, 1995.

Powell, R. M. & Puls, R. W. Proton Generation by Dissolution of Intrinsic or Augmented Aluminosilicate Ninerals

for In Situ Contaminant Generation by Zero-Valence-State Iron," *Environ. Sci. Technol.* 1997, 31 (8), 2244–2251.

Prins, van Nevel, Demeyer, "Pure culture studies of inhibitors for methanogenic bacteria," *Antonie van Leeuwenhoek,* 38(3):281–287, 1972.

Prokop and Bajpai, "Recombinant DNA Technology I: Conference on Progress in Recombinant DNA Technology and Application," Potosi, Mo., Jun. 3–8, 1990, *Annu. N.Y. Acad. Sci.,* 646:1–383, 1991.

Rajagopal and LeGall, "Utilization of cathodic hydrogen by hydrogen-oxidizing bacteria," *Appl. Microbiol. Biotechnol.,* 31:406–412, 1989.

Rawn, In: *Biochemistry,* Harper & Row Publishers, New York, 1983.

Reardon, *J. Environ. Sci. Technol.,* 29:2936–2945, 1996.

Rhodes and Carty, "The corrosion of certain metals by carbon tetrachloride," *Ind. Eng. Chem.,* 17:909–911, 1925.

Rickard and Fuerstran, "An electrochemical investigation of copper cementation by iron," *Trans. Metallurg. Soc. AIME,* XX:1487–1493, 1968.

Riley, Zachara, Wobber, "Chemical contaminants on DOE lands and selection of contaminant mixtures for subsurface science research," DOE/ER-0547T; U.S. DOE; Office of Energy Research, Washington, D.C., 1992.

Roberts, Totten, Arnold, Burris, Campbell, "Reductive elimination of chlorinated ethylenes by zero-valent metals," *Environ. Sci. Technol.,* 30(8):2654–2659, 1996.

Sambrook et al., "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989.

Scholz-Muramatsu, "Isolation and characterization of *Dehalospirillum multivorans* gen. nov, sp. nov, a tetrachloroethene-utilizing, strictly anaerobic bacterium," *Arch. Microbiol.,* 163:48–56, 1995.

Schuppler, Wagner, Schön and Göbel, "In situ identification of nocardioform actinomycetes in activated sludge using fluorescent rRNA-targeted oligonucleotide probes," *Microbiology,* 144:249–259, 1998.

Segal, In: *Biochemical Calculations,* 2nd Edition, John Wiley & Sons, New York, 1976.

Siegrist and McCarty, "Column methodologies for determining sorption and biotransformation potential for chlorinated aliphatic compounds in aquifers," *J. Contam. Hydrol.,* 2:31–50, 1987.

Sparling and Daniels, "The specificity of growth inhibition of methanogenic bacteria by bromoethanesulfonate," *Can. J. Microbiol.,* 33(12):1132–1136, 1987.

Spoerel and Kafatos, "Identification of genomic sequences corresponding to cDNA clones," *Methods Enzymol.,* 152:588–597, 1987.

Stinchcomb, Struhi, Davis, "Isolation and characterization of a yeast chromosomal replicator," *Nature,* 282(5734): 39, 1979.

Stromeyer, Stumpf, Cook, Leisinger, "Anaerobic degradation of tetrachloromethane by *Acetobacterium-Woodii* separation of dechlorinative activities in cell extracts and roles for vitamin B-12 and other factors," *Biodegradation,* 3(1):113–123, 1992.

Stromeyer, Winkelbauer, Kohler, Cook, Leisinger, "Dichloromethane utilized by an anaerobic mixed culture: acetogenesis and methanogenesis," *Biodegradation,* 2(2): 129–137, 1991.

Swanwick and Foulkes, "Inhibition of anaerobic digestion of sewage sludge by chlorinated hydrocarbons," *Water Pollut. Control,* 70:58–68, 1971.

Sweeny, "The reductive treatment of industrial wastewaters. II process applications," *AIChE Symp. Ser.,* 77:72–78, 1980.

Tatara, Dybas, Criddle, "Effects of medium and trace metals on kinetics of carbon tetrachloride transformation by psudomonas-SP strain KC," *Appl. Environ. Microbiol.,* 59(7):2126–2131, 1993.

Thiel, *Water Res.,* 3:215–223, 1969.

Thomas and Macaskie, "Biodegradation of tributyl phosphate by naturally occurring microbial isolates and coupling to the removal of uranium from aqueous solution," *Environ. Sci. Technol.,* 30:2371–2375, 1996.

Till B. A., L. J. Weathers, and P. J. J. Alvarez (1998). Fe^0-supported autotrophic denitrification. *Environ. Sci. Technol.,* 32(5), 634–639.

Trolard, Abdelmoula, Bourrié, Humbert and Génin, "Mise en évidence d'un constituant de type << rouilles vertes << dans les sols hydromorphes. Proposition de l'existence d'un nouveau minéral: la << fougérite >>," Trolard, Abdelmoula, Bourrié, Humbert and Génin," *C.R. Acad. Sci. Paris,* t. 323, série II a, p. 1015 à 1022, 1996.

Tschumper and Carbon, "Sequence of a yeast DNA fragment containing a chromosomal replicator and the TRP1 gene," *Gene,* 10(2):157–166, 1980.

Van Genutchen and Parker, "Boundary conditions for displacement experiments through short laboratory columns," *Soil Sci. Soc. Am. J.,* 48:703–708, 1984.

Vidic and Pohland, "Treatment Walls", *Groundwater Remediation Technologies Analysis Center,* Technology Evaluation Report TE-96-01. Uiniversity of Pittsburgh, Pittsburgh, Pa., October 1996.

Vogel et al., "Transformations of halogenated aliphatic compounds," *Environ. Sci. Technol.,* 21:722–736, 1987.

Wang, Mori, Komori, Sasatsu, Toda, Ohtake, "Isolation and characterization of an *Enterobacter cloacae* strain that reduces hexavalent chromium under anaerobic conditions," *Appl. Environ. Microbiol.,* 55(7):1665–1669, 1989.

Warwick, Bowen, McVeigh and Empley, "A phylogenetic analysis of the family Pseudonocardiaceae and the gnera Actinokineospora and Saccharothrix with 16S rRNA sequences and a proposal to combine the genera Amycolata and Pseudonocardia in an emended genus Pseudonocardia," *Int. J. Syst. Bacteriol.,* 44(2):293–299, 1994.

Weathers and Parkin, "Metallic iron-enhanced biotransformation of carbon tetrachloride and chloroform under methanogenic conditions," In: *Bioremediation of Chlorinated Solvents,* Hinchee et al. (Eds.), CRC Press, pp. 117–122, 1995.

Weathers and Parkin, In: *Bioremediation of Chlorinated Solvents,* Hinchee R E, Leeson A, Semprini L, (Eds.), CRC Press, Boca Raton, Fla., pp. 117–122, 1995.

Weathers, Parkin, Novak, and Alvarez, "Chloroform cometabolism by a methanogenic consortium utilizing zero-valent iron as ultimate electron donor," *Abst. Gen. Meet. Am. Soc. Microbiol.,* 95(0):399, 1995a.

Weathers, Parkin, Novak, and Alvarez, "Methanogens couple anaerobic Fe(0) oxidation and $CHCl_3$ reduction," *Abst. Papers Am. Chem. Soc.,* 209(1–2):ENVR225, 1995b.

Weathers, Ph.D. Dissertation, University of Iowa, 1995.

Wild, Winkelbauer, Leisinger, "Anaerobic dechlorination of trichloroethene, tetrachloroethene and 1,2-dichloroethane by an acetogenic mixed culture in a fixed-bed reactor," *Biodegradation,* 6(4):309–318, 1995.

Wong and Neumann, "Electric field mediated gene transfer," *Biochim. Biophys. Res. Commun.,* 107(2):584–587, 1982.

Yamamoto et al., "Kinetics and modeling of hexavalent chromium reduction in *Enterobacter cloacae*," *Biotechnol. Bioeng.*, 41:129–133, 1993.

Yang and Speece, "The effects of chloroform toxicity on methane fermentation," *Water Res.*, 20(10):1273–1280, 1986.

Zhang, Liu, Logan, Mazumnder and Phelps, "Enhancement of Fe(III), Co(III), and Cr(VI) reduction at elevated temperatures and by a thermophilic bacterium," *Appl. Biochem. Biotech.*, 57/58:923–932, 1996.

Zinder, Anguish, Cardwell, "Selective inhibition by 2 bromo ethanesulfonate of methanogenesis from acetate in a thermophilic anaerobic digestor," *Appl. Environ. Microbiol.*, 47(6):1343–1345, 1984.

Zinder, In: *Methanogenesis,* Ferry (Ed.), Chapman and Hall, New York, pp. 128–206, 1993.

All of the compositions, methods and apparatus disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions, methods and apparatus and in the steps or in the sequence of steps of the methods described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

What is claimed is:

1. A device comprising a composition comprising one or more autotrophic hydrogenotrophic bacteria in culture medium comprising zero-valent iron, wherein said device is comprised within a water-, wastewater- or sewage-treatment system or a system for remediating pollution in an aqueous solution or an environmental site.

2. The device in accordance with claim **1**, wherein said hydrogenotrophic bacteria comprise one or more species of bacteria selected from the group consisting of Acetobacterium spp., Achromobacter spp., Aeromonas spp., Acinetobacter spp., Aureobacterium spp., Bacillus spp., Comamonas spp., Dehalobacter spp., Dehalospirillum spp., Dehalococcoide spp., Desulfurosarcina spp., Desulfomonile spp., Desulfobacterium spp., Enterobacter spp., Hydrogenobacter spp., Methanosarcina spp., Pseudomonas spp., Shewanella spp., Methanosarcina spp., Micrococcus spp., and Paracoccus spp.

3. The device of claim **2**, wherein said hydrogenotrophic bacteria comprise one or more strains of bacteria selected from the group consisting of *Acetobacterium woodi, Aeromonas hydrophila, Aeromonas sobria, Alcaligenes eutrophus, Comamonas acidovorans, Dehalococcoide restrictus, Dehalococcoide multivorans, Dehalococcoide ethenogene, Desulfobacterium tiedje, Enterobacter agglomerans, Hydrogenobacter thermophilus, Methanosarcina barkeri, Methanosarcina mazei, Methanosarcina thermophila, Paracoccus denitrificans, Pseudomonas aureofaciens, Pseudomonas maltophilia, Pseudomonas mendocina,* and *Shewanella putrefaciens.*

4. The device of claim **3**, wherein said hydrogenotrophic bacteria comprise *Paracoccus denitrifcans* ATCC17741, *Paracoccus denitrificans* ATCC35512, *Paracoccus denitrificans* ATCC 13543, or *Paracoccus denitrificans* ATCC 19367.

5. The device of claim **1**, wherein said zero-valent iron comprises Fe(0) metal, an Fe(0) alloy, or an Fe(0)—Ni(0), Fe(0)—Zn(0), Fe(0)—Pt(0), or Fe(0)—Pd(0) bimetal.

6. The device of claim **5**, wherein said zero-valent iron comprises filings, shavings, turnings, wool, powder, mesh, beads, rods, pellets, or flakes.

7. The device of claim **1**, further comprising a support.

8. The device of claim **7**, further comprising a glass, concrete, metallic, zeolite, mineral, fiber, fiberglass, ceramic, plastic, polymeric, or resin support.

9. The device of claim **1**, further defined as an ex situ bioreactor.

10. The device in accordance with claim **9**, comprising an inlet port, an outlet port and a container means for containing said composition.

11. The device of claim **10**, further defined as a continuous culture system, a flow-through packed column, an inline water filter, a biofermenter, a fluidized bed, a sequencing batch reactor, or an anaerobic digester.

12. The device of claim **1**, comprised within a water-, wastewater- or sewage-treatment system.

13. The device in accordance with claim **12**, comprised within a water treatment system, a sewage or wastewater treatment system, a municipal water supply system, or a pollution decontamination system.

14. The device of claim **1**, comprised within a system for remediating pollution in an aqueous solution or an environmental site.

15. A device comprising a composition comprising one or more autotrophic hydrogenotrophic bacteria in culture medium comprising zero-valent iron, said device being comprised within an environmental site.

16. The device of claim **15**, comprised within a landfill site, an agricultural site, an agricultural runoff site, or an irrigation site.

17. The device of claim **15**, further defined as an in situ reactive barrier.

18. The device in accordance with claim **17**, further defined as a permeable barrier, a semipermeable barrier, a treatment wall, and injected treatment zone, or a funnel and gate system.

19. A method of removing or reducing the concentration of an organic or inorganic compound in an environmental site, comprising providing to said site an effective amount of a composition comprising one or more hydrogenotrophic bacteria and zero-valent iron, or contacting said site with a device comprising a composition comprising culture medium comprising one or more hydrogenotrophic bacteria and zero-valent iron.

20. A method for denitrifying groundwater or an environmental site in situ comprising contacting said groundwater or said environmental site with a composition comprising zero-valent iron and a culture of one or more hydrogenotrophic bacteria, or contacting said site with a device comprising a composition comprising zero-valent iron and a culture of one or more hydrogenotrophic bacteria.

21. A method for removing or reducing the concentration of a nitrogen- or sulfur-containing compound in a sample, comprising contacting a sample suspected of containing said compound with a composition comprising zero-valent iron and a culture of one or more hydrogenotrophic bacteria, or contacting said site with a device comprising a composition comprising zero-valent iron and a culture of one or more hydrogenotrophic bacteria.

22. The method in accordance with claim **21**, wherein said sulfur-containing compound is sulfate or sulfite.

23. A method for removing or reducing the concentration of a halocarbon compound in a sample, comprising contact-

ing a sample suspected of containing said halocarbon with a composition comprising one or more autotrophic hydrogenotrophic bacteria and zero-valent iron, or contacting said sample with a device comprising a composition comprising culture medium comprising one or more autotrophic hydrogenotrophic bacteria and zero-valent iron.

24. The method in accordance with claim **23**, wherein said halocarbon is carbon tetrachloride, dichloromethane, a polychlorinated biphenyl, a chlorinated benzene, trichloroethylene, perchloroethylene, dichloroethylene, vinyl chloride, chloroethane, bromoform, dichlorodifluoromethane, trihalomethanes, tetrachlorodibenzodioxin pentachlorophenol, a chlorobenzoate, atrazine, or 1,1,1-TCA.

25. The method of claim **24**, wherein said halocarbon is carbon tetrachloride, dichloromethane, trichloroethylene, perchloroethylene, dichloroethylene, vinyl chloride, chloroethane, dichlorodifluoromethane, trihalomethanes, tetrachlorodiberzodioxin pentachlorophenol, a chlorobenzoate, atrazine, or 1,1,1-TCA.

26. The method of claim **25**, wherein said halocarbon is carbon tetrachloride, trichloroethylene, or dichloromethane.

27. A method for removing or reducing the concentration of a haloaromatic compound in a sample, comprising contacting a sample suspected of containing said haloaromatic compound with a composition comprising zero-valent iron and a culture of one or more hydrogenotrophic bacteria, or contacting said sample with a device comprising a composition comprising zero-valent iron and a culture of one or more hydrogenotrophic bacteria.

28. The method in accordance with claim **27**, wherein said haloaromatic compound is a polychlorinated biphenyl, a chlorinated benzene, tetrachlorodibenzodioxin pentachlorophenol, a chlorobenzoate, atrazine, or 1,1,1-TCA.

29. A method for degrading or detoxifying a pesticide, comprising contacting a sample suspected of containing said pesticide with a composition comprising zero-valent iron and a culture of one or more hydrogenotrophic bacteria, or contacting said sample with a device comprising a composition comprising zero-valent iron and a culture of one or more hydrogenotrophic bacteria.

30. The method in accordance with claim **29**, wherein said pesticide is methoxyclor, alachlor, metolachlor, lindane, DDT, DDE, DDD, dieldrin, aldrin, heptachlor, chlordane, 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid or atrazine.

31. The method of claim **30**, wherein said pesticide is atrazine.

32. A method for detoxifying a metal ion-containing compound, comprising contacting a sample suspected of containing said compound with a composition comprising zero-valent iron and a culture of one or more hydrogenotrophic bacteria, or contacting said sample with a device comprising a composition comprising zero-valent iron and a culture of one or more hydrogenotrophic bacteria.

33. The method in accordance with claim **32**, wherein said compound comprises strontium (II), cesium (I), chromium (VI) uranium (VI), technetium (VII), silver (I), or mercury (II).

34. The method of claim **33**, wherein said compound comprises chromium (VI) or uranium (VI).

35. A method for reducing the concentration of nitrite-, nitrate-, sulfite-, or sulfate-containing compound in an aqueous solution or environmental site, comprising (a) selecting an aqueous solution or an environmental site containing said compound; and (b) contacting said solution or site with a composition comprising zero-valent iron and a culture of one or more hydrogenotrophic bacteria, or contacting said solution or site with a device comprising a composition comprising zero-valent iron and a culture of one or more hydrogenotrophic bacteria.

36. A method for reducing the concentration of a pesticide or organic pollutant in an aqueous solution or environmental site, comprising (a) selecting an aqueous solution or an environmental site containing said pesticide or pollutant; and (b) contacting said solution or site with a composition comprising zero-valent iron and a culture of one or more autotrophic hydrogenotrophic bacteria, or contacting said solution or site with a device comprising a composition comprising zero-valent iron and a culture of one or more autotrophic hydrogenotrophic bacteria.

37. A method for reducing the concentration of a mercury-, silver-, technetium-, strontium-, cesium-, chromium- or uranium-containing pollutant in an aqueous solution or environmental site, comprising (a) selecting an aqueous solution or an environmental site containing said pollutant; and (b) contacting said solution or site with a composition comprising zero-valent iron and a culture of one or more hydrogenotrophic bacteria, or contacting said solution or site with a device comprising a composition comprising zero-valent iron and a culture of one or more hydrogenotrophic bacteria.

38. A method for reducing silver (I), mercury (II), technetium (VII), strontium (II), cesium (I), chromium (VI) or uranium (VI) ions in an aqueous solution, comprising contacting an aqueous solution suspected of containing one or more of said ions with a composition comprising zero-valent iron and a culture of one or more hydrogenotrophic bacteria, or contacting said aqueous solution with a device comprising a composition comprising zero-valent iron and a culture of one or more hydrogenotrophic bacteria.

39. A method for removing or reducing the concentration of a nitroaromatic compound in a sample comprising contacting a sample suspected of containing said nitroaromatic compound with one or more hydrogenotrophic bacteria and zero-valent iron, or a device comprising culture medium comprising zero-valent iron.

40. The method in accordance with claim **39**, wherein said nitroaromatic compound is trinitrotoluene, RDX, HMX, 2-aminodintrotoluene, 4-aminodinitrotoluene, or parathion.

41. The method in accordance with claim **39**, wherein said nitroaromatic compound is trinitrotoluene, RDX, or HMX.

* * * * *

US007105488B1

(12) **United States Patent**
Tarasova et al.

(10) **Patent No.: US 7,105,488 B1**
(45) **Date of Patent: Sep. 12, 2006**

(54) **G PROTEIN-COUPLED RECEPTOR ANTAGONISTS**

(75) Inventors: **Nadya I. Tarasova**, Frederick, MD (US); **Christopher J. Michejda**, North Potomac, MD (US)

(73) Assignee: **The United States of America as represented by the Department of Health and Human Services**, Washington, DC (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/601,600**

(22) PCT Filed: **Feb. 26, 1999**

(86) PCT No.: **PCT/US99/04438**

§ 371 (c)(1),
(2), (4) Date: **Oct. 2, 2000**

(87) PCT Pub. No.: **WO99/43711**

PCT Pub. Date: **Sep. 2, 1999**

Related U.S. Application Data

(60) Provisional application No. 60/076,105, filed on Feb. 27, 1998.

(51) **Int. Cl.**

A61K 38/04	(2006.01)
C07K 5/00	(2006.01)
C07K 16/00	(2006.01)
C07K 17/00	(2006.01)
G01N 33/567	(2006.01)

(52) **U.S. Cl.** **514/16**; 514/2; 514/17; 514/18; 514/19; 530/328; 530/29; 530/30; 536/23.5; 435/7.21; 436/501

(58) **Field of Classification Search** 514/2, 514/12, 13, 14; 530/300, 324, 326, 327, 530/332, 345, 350; 435/7.21, 69.1, 69.2, 435/69.4; 436/501; 536/23.5
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,508,384 A 4/1996 Murphy et al.

FOREIGN PATENT DOCUMENTS

WO	WO 94/05695	*	3/1994
WO	WO 97/28258		8/1997
WO	WO 97/35881		10/1997
WO	WO 98/00538		1/1998

OTHER PUBLICATIONS

Ulrich et al., Biochem. Biophys. Res. Comm. 193(1)204-211, 1993.*
Bowie et al., 1990, Science 247:1306-1310.*
Guo-HH et al. PNAS 101(25)9205-9210, 2004.*
Anand-Srivastava et al. (1996) "Cytoplasmic Domain of Natiuretic Peptide Receptor-C Inhibits Adenylyl Cyclase", J. Biol.Chem. 271:19324-19329.
Gudermann et al. (1997) "Functional and structural complexity of signal transduction via G-protein-coupled receptors", Annu. Rev. Neurosci. 20:399-427.
Hebert et al. (1996) "A peptide derived from a beta2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation", J. Biol. Chem. 71(27):16384-92.
Merkouris et al. (1996) "Identification of the critical domains of the delta-opioid receptor involved in G protein coupling using site-specific synthetic peptides", Mol. Pharmacol. 50(4):985-93.
Monnot et al. (1996) "Polar residues in the transmembrane domains of the type 1 angiotensin II receptor are required for binding and coupling. Reconstitution of the binding site by co-expression of two deficient mutants", J. Biol. Chem. 271(3):1507-13.
Moro et al. (1993) "Hydrophobic amino acid in the i2 loop plays a key role in receptor -G protein coupling", J. Biol. Chem. 268:22273-6.
Osuga et al. (1997) "Co-expression of defective luteinizing hormone receptor fragments partially reconstitutes ligand-induced signal generation", J. Biol. Chem. 272:25006-12.
Raport et al. (1996) "Molecular cloning and functional characterization of a novel human CC chemokine receptor (CCR5) for RANTES, MIP-1beta, and MIP-1alpha", J. Biol. Chem. 271:17171-17166.
Schoneberg et al. (1996) "Functional rescue of mutant V2 vasopressin receptors causing nephrogenic diabetes insipidus by a co-expressed receptor polypeptide", EMBO J. 15:1283-91.
Wong et al. (1990) "Chimeric muscarinic cholinergic: beta-adrenergic receptors that activate Gs in response to muscarinic agonists", J. Biol. Chem. 265:6219-6324.

* cited by examiner

Primary Examiner—Janet L. Andres
Assistant Examiner—Michael Brannock
(74) *Attorney, Agent, or Firm*—Townsend and Townsend and Crew LLP

(57) **ABSTRACT**

G-protein coupled receptors (GPCR) generally contain seven transmembrane helices. The present invention provides synthetic peptides derived from these transmembrane helices. The peptides inhibit GPCR function by disrupting GPCR structure. In certain embodiments, charged residues are added at one terminus to promote correct orientation of the peptide in the membrane.

7 Claims, 3 Drawing Sheets

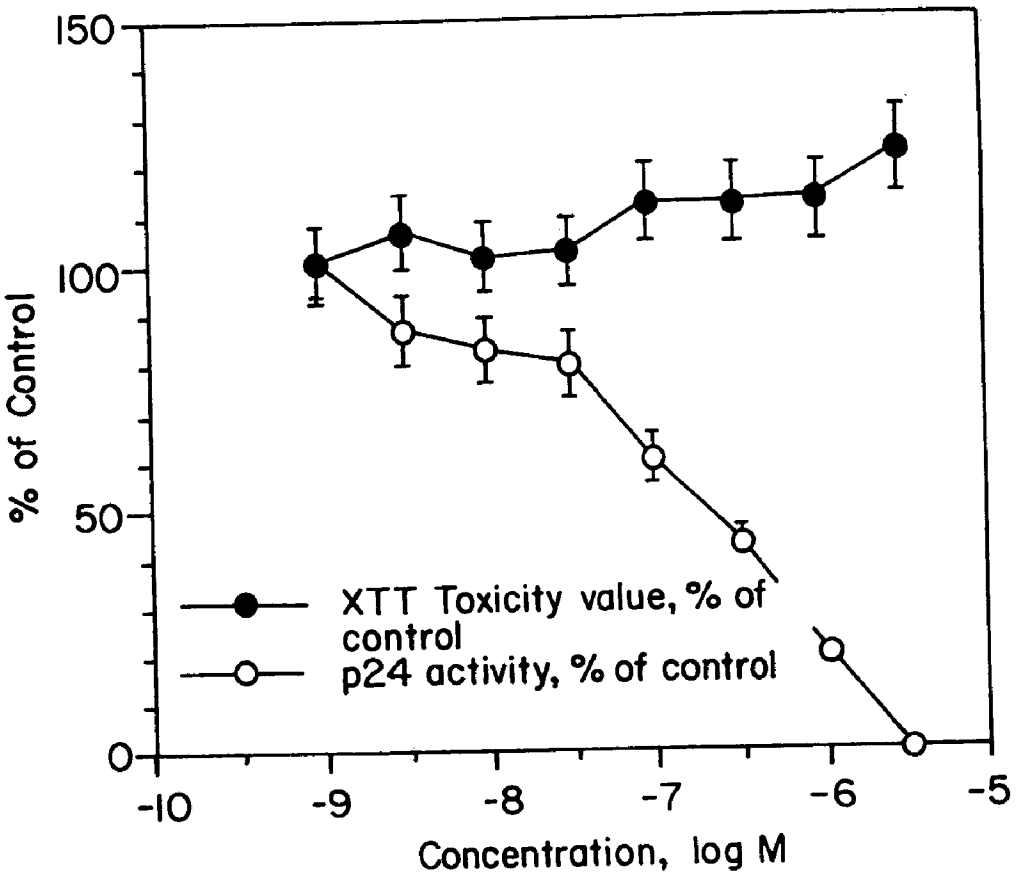

150
100
50
0
% of Control
-10
-9
-8
-7
-6
-5
Concentration, log M
XTT Toxicity value, % of control
p24 activity, % of control

FIG. 1.

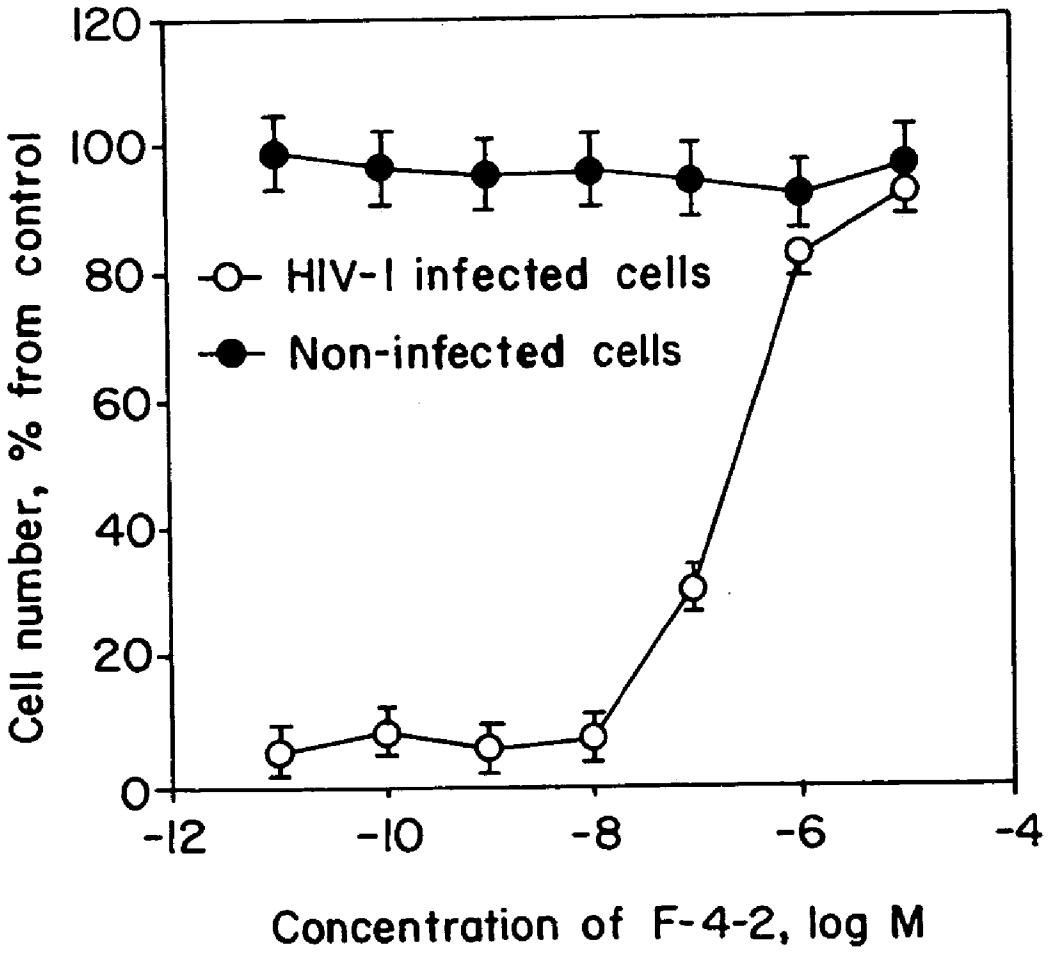

120
100
80
60
40
20
0
Cell number, % from control
HIV-I infected cells
Non-infected cells
-12
-10
-8
-6
-4
Concentration of F-4-2, log M

FIG. 2.

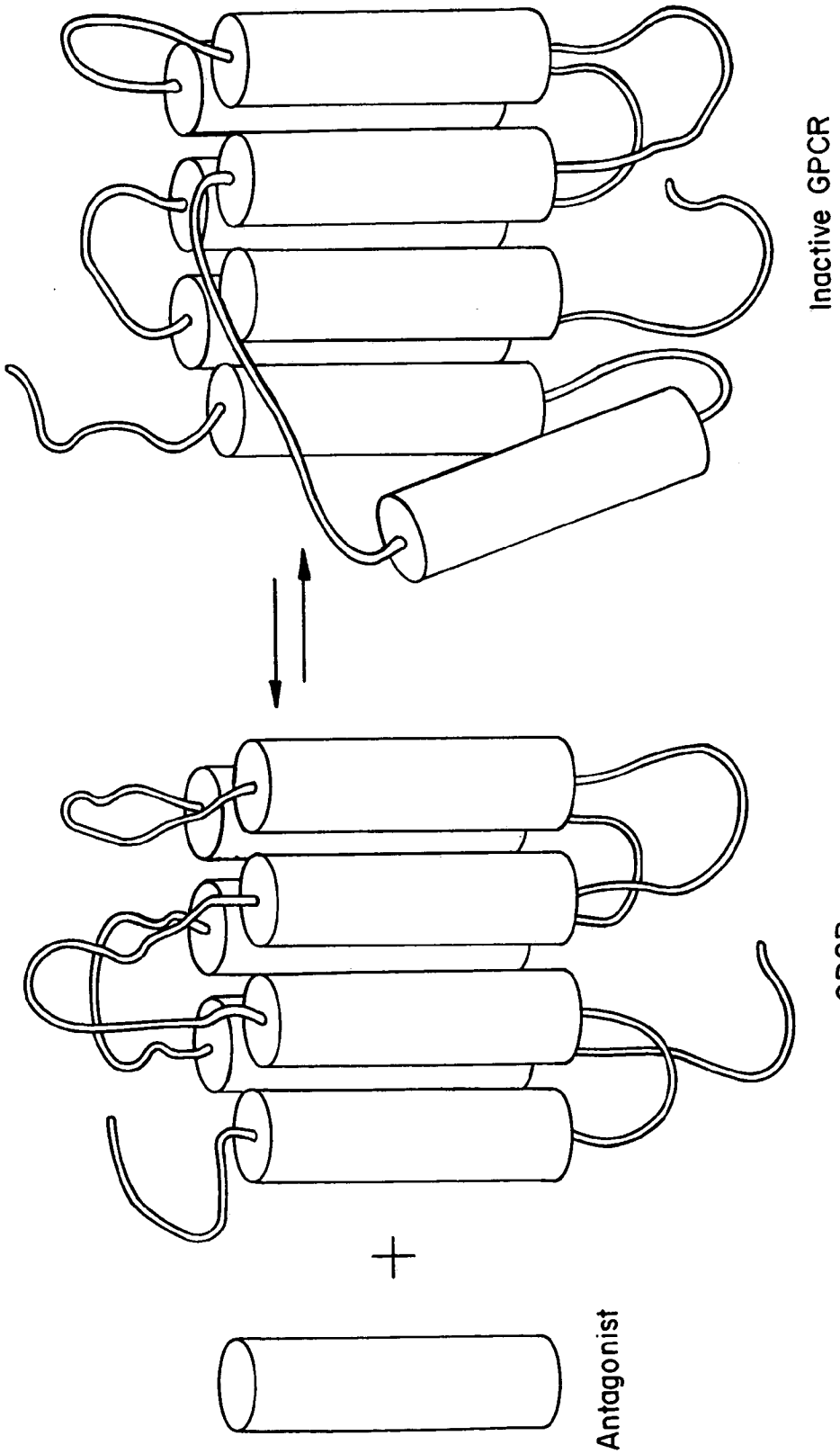


FIG. 3.

G PROTEIN-COUPLED RECEPTOR ANTAGONISTS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/076,105 filed Feb. 27, 1998, the disclosure of which is incorporated by reference.

FIELD OF THE INVENTION

The present invention relates to modulating, especially inhibiting, biological activities of G protein coupled receptors (GPCRs) by exposing GPCRs to molecules which interfere with correct receptor assembly. In particular, the invention relates to synthetic, isolated and/or recombinant peptides, fragments and/or consensus peptides of the transmembrane domain of GPCRs that inhibit GPCR-mediated signal transduction.

BACKGROUND OF THE INVENTION

Many physiologically important events are mediated by the binding of guanine nucleotide-binding regulatory proteins (G proteins) to G protein-coupled receptors (GPCRs). These events include vasodilation, stimulation or decrease in heart rate, bronchodilation, stimulation of endocrine secretions and enhancement of gut peristalsis, development, mitogenesis, cell proliferation and oncogenesis.

G proteins are a diverse superfamily of guanine nucleotide-binding proteins that play a central role in signal transduction and regulation of cellular metabolism. They are generally comprised of three subunits: a guanyl-nucleotide binding alpha subunit; a beta subunit; and a gamma subunit. (For a review, see Conklin et al. *Cell* 73, 631–641, (1993)). G proteins commonly cycle between two forms, depending on whether GDP or GTP is bound to the alpha subunit. When GDP is bound, the G protein exists as a heterotrimer, the G alpha-beta-gamma complex. When an alpha-beta-gamma complex operatively associates with a ligand-activated GPCR in a cell membrane, the rate of exchange of GTP for bound GDP is increased and the G alpha subunit dissociates from the G beta-gamma complex. The free G alpha subunit and G beta-gamma complex are capable of transmitting a signal to downstream elements of a variety of signal transduction pathways, for example by binding to and activating adenyl cyclase. This fundamental scheme of events forms the basis for a multiplicity of different cell signaling phenomena.

Recent studies have suggested that all members of the GPCR superfamily have a conserved structure. Comparisons of avian and mammalian beta-adrenergic receptor cDNA's (Yarden et al., *Proc. Natl. Acad. Sci. USA* 83: 6795–6799, 1986; Dixon et al., *Nature* 321:75–79, 1986; and Kobilka et al., *Proc. Natl. Acad. Sci. USA* 84:46–50, 1987), a bovine rhodopsin cDNA (Nathans and Hogness, *Cell* 34:807–814, 1983), an alpha 2-adrenergic receptor (Kobilka et al., *Science* 238:650–656, 1987), an angiotensin receptor cDNA (Young et al., *Cell* 45:711–719, 1986; Jackson et al., *Nature* 335:437439, 1988), a bovine substance K receptor (Masu et al., *Nature* 329:836–838, 1987), and a muscarinic acetylcholine receptor cDNA (Kubo et al., *Nature* 323:411416, 1986) predict that all GPCR share a highly conserved presence of seven hydrophobic transmembrane domains that are suggested to be transmembrane helices of 20–30 amino acids connected by extracellular or cytoplasmic loops.

Kobilka et al., *Science* 240: 1310 (1988); Maggio et al., *FEBS Lett.* 319: 195 (1993); Maggio et al., *Proc. Natl. Acad. Sci USA* 90: 3103 (1993); Ridge et al., *Proc. Natl. Sci USA* 91, 3204 (1995); Schonenberg et al., *J. Biol. Chem.* 270: 18000 (1995); Huang et al., *J. Biol. Chem.* 256: 3802 (1981); Popot et al., *J. Mol. Biol.* 198: 655 (1987); Kahn and Engelman, *Biochemistry* 31: 6144 (1992); Schoneberg et al. *EMBO J.* 15: 1283 (1996); Wong et al., *J. Biol. Chem.* 265: 6219 (1990); Monnot et al., *J. Biol. Chem.* 271: 1507 (1996); Gudermann et al., *Annu. Rev. Neurosci.* 20: 399 (1997); Osuga et al., *J. Biol. Chem.* 272: 25006 (1997); Lefkowitz et al., *J. Biol. Chem.* 263:4993–4996, 1988; Panayotou and Waterfield, *Curr. Opinion Cell Biol.* 1:167–176, 1989. These transmembrane domains of G-protein coupled receptors are designated TM1, TM2, TM3, TM4, TMS, TM6 and TM7. TM4, TM5, TM6 and TM7 are the most highly conserved and are postulated to provide sequences which impart biological activity to GPCRs. TM3 is also implicated in signal transduction.

The coupling of GPCRs to intracellular signaling molecules such as adenylate cyclase (Anand-Srivastava et al., *J. Biol. Chem.* 271: 19324–19329 (1996)) and G-proteins (Merkouris et al., *Mol. Pharmacol.* 50: 985–993 (1996)) is reportedly inhibited by peptides corresponding to the intracellular loops of the receptors. Those studies were conducted primarily to provide an understanding of molecular mechanisms of receptor function and could not be applied directly for drug design, because of the difficulties in intracellular delivery of the inhibitors.

WO 94/05695 and U.S. Pat. No. 5,508,384 set forth sequences of transmembrane regions for 74 GPCRs. The WO 94/05695 patent publication describes and claims polypeptides corresponding to fragments or homologous sequences of GPCRs which can bind a GPCR ligand or which can modulate ligand binding. Both references disclose that a membrane spanning fragment of the third TM domain of the dopamine D_2 receptor specifically bound a ligand of the intact receptor in a simple, small unilamellar vesicle model. The fragment used was terminated with a lysine (which is positively charged at physiological pH) at one end and with an aspartic acid (which is negatively charged at physiological pH) at the other. This peptide would not be expected to insert readily into a biological membrane.

SUMMARY OF THE INVENTION

The invention generally comprises peptide or peptidomimetic compounds that modulate, and preferably inhibit the biological properties and activities of GPCRs, by targeting the transmembrane portions of these receptors. The present invention specifically comprises methods for disrupting GPCR function by using these GPCR antagonists.

The present invention provides for the use of chemical or recombinant DNA technology to obtain GPCR polypeptides, which preferably are as small as possible while still retaining sufficiently high affinity for binding to, or association with, GPCRs. Non-limiting examples of GPCR polypeptides include fragments of 10 to 50 amino acids corresponding to at least one transmembrane domain of domains 1–7. The following are nonlimiting examples of GPCR peptides with antagonist properties.

From the GPCR CXCR4
F-2-2: LLFVITLPFWAVDAVANWYFGNDD (SEQ ID NO:1)

F-2-5: LLFVITLPFWAVDAVANDD (SEQ ID NO:2)

F-4-2: VYVGVWIPALLLTIPDFIFANDD (SEQ ID NO:3)

F-6-1: VILILAFFACWLPYYIGISID (SEQ ID NO:4)

F-7-3: DDEALAFFHCCLNPILYAFL-NH_2 (SEQ ID NO:5)

F-7-4: DDSITEALAFFHCCLNPILYAFL-NH_2 (SEQ ID NO:6)

From the GPCR CCR5
CCR5-TM-2-2: LFFL LTVPFWAHYAAAQWDFGDD (SEQ ID NO:7)

CCR5-TM-4-1: FGVVTSVITWVVAVFASLPGIIFTSSDD (SEQ ID NO:8)

CCR5-TM-6-1: LIFTIMIVYFLFWAPYNIVLLLNTFQED (SEQ ID NO:9)

CCR5-TM-7-1: DDQAMQVTETLGMTHCCINPIIYAFV (SEQ ID NO:10)

From the GPCR CCR2
CCR2-TM-2-1: IYLLNLAISDLLFLITLPLWADD (SEQ ID NO:11)

CCR2-TM-2-2: LLFLITLPLWAH SAANEWVFGNDD (SEQ ID NO:12)

CCR2-TM-4-1: FGVVTSVITWLVAVF ASVPGIIFTDD (SEQ ID NO:13)

CCR2-TM-6-1: VIFTIMIVYFLFWTPYN IVILLNTFQED (SEQ ID NO:14)

CCR2-TM-7-1: DDATQVT ETLGMTHCCINPIIYAFV (SEQ ID NO:15)

From the GPCR CCR3
CCR3-TM-2-1: LLFLVTLPFW IHYVRGHNWVFGDDD (SEQ ID NO:16)

CCR3-TM-4-1: FGVITSIVTWGLAVLAALPEFI FYETED (SEQ ID NO:17)

CCR3-TM-6-1: IFVIMAVFFI FWTPYNVAILLSSYQSDD (SEQ ID NO:18)

CCR3-TM-7-1: DDLVMLVTEVIAYSHCCMNPVIYAFV (SEQ ID NO:19)

From the GPCR CCKAR
CCKAR-TM-1-6: DDEWQSALQILLYSIIFLLSV-LGNTLVITV (SEQ ID NO:20)

CCKAR-TM-2-1: FLLSLAVSDLMLCLFCMPFNLP (SEQ ID NO:21)

CCKAR-TM-2-2: FLLSLAVSDLMLCLFCM PFNLIDD (SEQ ID NO:22)

CCKAR-TM-6-4: IVVLFFLCWMPIFSANAWRAYDTVDD (SEQ ID NO:23)

One embodiment of the invention is an isolated G protein-coupled receptor (GPCR)-modulating molecule comprising a peptide or peptidomimetic that is a structural analog of a portion of a transmembrane domain of a GPCR, wherein said molecule has a first end and a second end and said molecule has at said first end a negatively charged group and at said second end a neutral charge under physiological conditions; said molecule spontaneously inserts into a membrane in the same orientation as the transmembrane domain from which it is derived; and said molecule modulates a biological property or activity of said GPCR.

In a particular embodiment, the molecules contain a hydrophilic, negatively charged non-peptidic head group and an uncharged tail, which assures correct orientation of the molecule in the cell membrane. In another embodiment, the negatively charged head group is one or more acidic amino acids.

Another embodiment is an isolated GPCR-modulating molecule comprising a peptide or peptidomimetic that is a structural analog of a portion of a transmembrane domain of CXCR4, wherein said portion of said transmembrane domain has a sequence selected from the group of sequences consisting of:

LLFVITLPFWAVDAVANWYFGNDD (SEQ ID NO:1),

LLFVITLPFWAVDAVANDD (SEQ ID NO:2),

VYVGVWIPALLLTIPDFIFANDD (SEQ ID NO:3),

VILILAFFACWLPYYIGISID (SEQ ID NO:4),

DDEALAFFHCCLNPILYAFL-NH_2 (SEQ ID NO:5),

DDSITEALAFFHCCLNPILYAFL-NH_2 (SEQ ID NO:6),

wherein said molecule modulates a biological activity of said CXCR4. The CXCR4 activity modulated by said peptide includes inhibition of CXCR4-mediated intracellular Ca^{2+} release and inhibition of CXCR4-mediated HIV infection.

The invention also comprises methods of modulating the biological activity of a target GPCR by contacting a cell that expresses said GPCR with a molecule of the invention. In

one method, the target GPCR is CXCR4, CCR5 or CCR2, and the modulated biological activity is inhibition of GPCR-mediated HIV infection. In another method, the target GPCR is CXCR4 and the modulated biological activity is inhibition of CXCR4-mediated intracellular Ca^{2+} release.

Another embodiment is a method of inhibiting HIV-1 infection, comprising contacting a cell that expresses a GPCR that binds HIV-1 with a molecule that comprises a peptide or peptidomimetic that is a structural analog of a portion of a transmembrane domain of said GPCR, wherein contacting the cell with said molecule inhibits HIV-1 infection. The peptide or peptidomimetic may be a structural analog of a portion of a transmembrane domain of CXCR4 or CCR5. Peptides corresponding to TM regions of CXCR4, a GPCR that functions as a co-receptor during the cell entry of HIV, were designed and tested in cells, and yielded potent inhibition of HIV entry without apparent toxicity to the cells.

The usefulness of the method is demonstrated by specifically targeting the CXCR4 that functions as a co-receptor during the cell entry of T-cell tropic strains of HIV-1. Peptides containing 20–25 amino acid residues inhibited receptor signaling and HIV-1 infection in vitro at concentration as low as 0.2 micromolar.

In one embodiment, the molecules of the present invention mimic a transmembrane domain of the chosen receptor and block self-assembly of that receptor, possibly by competitive inhibition with the native TM domain. They thereby block or inhibit signal transduction in the affected cell.

The invention also includes peptide analogs and peptidomimetics which possess beneficial properties such as increased half-life, lack of immunogenicity, and the ability to cross the blood-brain barrier.

The peptide analogs of the invention mediate the chemical and/or biological effects of hormone agonists/antagonists or other peptides. They are believed to be useful for the development of pharmaceutical, therapeutic, and diagnostic techniques. Accordingly, the invention also provides methods for producing a prophylactic or therapeutic response in a mammal by administering to the mammal a pharmaceutically effective amount of one or more peptide analogs of the invention. In preferred embodiments, the present invention provides methods for producing such responses by modulating the activity of at least one mammalian G-protein-linked receptor by administering an effective amount of one or more peptide analogs of the invention.

In another embodiment, a peptide of the invention may modulate the biological activity of more than one GPCR. In another embodiment, more than one peptide of the invention are administered as a cocktail to modulate the biological activity of more than one GPCR.

BRIEF DESCRIPTION OF FIGURES

FIG. **1**. Anti-HIV efficacy and toxicity of F-2-2 assay. CEM-SS cells were infected with the LAV strain of HIV-1.

FIG. **2**. Anti-HIV efficacy and toxicity of F-4-2 in cytoprotection. CEM-SS cells were infected with the RF strain of HIV-1, which causes cell death, if the inhibitor of infection is not present.

FIG. **3**. The proposed model of transmembrane antagonists action.

DEFINITIONS

A "G-protein" is any member of the superfamily of signal transducing guanine nucleotide binding proteins.

A "G-protein-coupled receptor" is any member of a superfamily of receptors that mediates signal transduction by coupling with a G protein. Examples of such receptors include, but are not limited to: CC chemokine receptor 5 (CCR5), CXC chemokine receptor (CXCR4) cholecystokinin type A receptor (CCKAR), adenosine receptors, somatostatin receptors, dopamine receptors, muscarinic cholinergic receptors, alpha-adrenergic receptors, beta-adrenergic receptors, opiate receptors, cannabinoid receptors, growth hormone releasing factor, glucagon, cAMP receptors, serotonin receptors (5-HT), histamine H2 receptors, thrombin receptors, kinin receptors, follicle stimulating hormone receptors, opsins and rhodopsins, odorant receptors, cytomegalovirus GPCRs, histamine H2 receptors, octopanmine receptors, N-formyl receptors, anaphylatoxin receptors, thromboxane receptors, IL-8 receptors, platelet activating factor receptors, endothelin receptors, bombesin gastrin releasing peptide receptor, neuromedin B preferring bombesin receptors, vasoactive intestinal peptide receptors, neurotensin receptors, bradykinin receptors, thyrotropin-releasing hormone receptors, substance P receptors, neuromedin K receptors, renal angiotensin II type I receptors, mas oncogene (angiotensin) receptors lutropin-choriogonadotropin receptors, thyrotropin receptors, follicle stimulating hormone receptors, cannabinoid receptors, glucocorticoid-induced receptors, endothelial cell GPCRs, testis GPCRs, and thoracic aorta GPCRs, and homologs thereof having a homology of at least 80% with at least one of transmembrane domains 1–7, as described herein. See, e.g., Probst et al, *DNA and Cell Biology* 11: 1–20 (1992), which is entirely incorporated herein by reference. The term further encompasses subtypes of the named receptors, and mutants and homologs thereof, along with the DNA sequences encoding the same.

The term "membrane" refers generally to a lipid bilayer. Preferably, the lipid bilayer is the plasma membrane that delimits a cell, but may be any cellular membrane. The term membrane also encompasses bilayer structures, such as artificial liposomes.

The term "GPCR polypeptide" includes polypeptides having an amino acid sequence which substantially corresponds to at least one 10 to 50 (e.g., 10, 20, 25 30 residues) amino acid fragment and/or homologous sequence of a known GPCR or group of GPCRs, wherein the GPCR polypeptide has homology of at least 80%, such as 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92 93, 94, 95, 96, 97, 98, 99 or 100% homology, while maintaining GPCR modulating activity, wherein a GPCR polypeptide of the present invention is not naturally occurring or is naturally occurring but is in a purified or isolated form which does not occur in nature.

Preferably, a GPCR polypeptide of the present invention substantially corresponds to a transmembrane domain of a GPCRs. Also preferred are GPCR polypeptides wherein the GPCR amino acid sequence is 4–10 to 50 amino acids in length, such as 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 amino acids, or any range therein.

The term "spontaneously inserts into a membrane" means that a peptide that is brought into contact with a membrane will, under physiological conditions, arrange itself within the lipid bilayer such that the hydrophobic portion of the peptide is within the membrane, and any charged end is exposed to either surface of a membrane. Preferably, molecules of the present invention that have a net negative

charge at one end will orient themselves so that the charged end faces the extracellular surface of the cell.

The term "tumor cell" or "cancer cell" or "neoplastic cell" denotes a cell that demonstrates inappropriate, unregulated proliferation. A cell line is said to be "malignant" if, when the cell line is injected into a host animal, the host animal develops tumors or cancers that are anaplastic, invasive, and/or metastatic. A "human" tumor is comprised of cells that have human chromosomes. Such tumors include those in a human patient, and tumors resulting from the introduction of a human malignant cell line into a non-human host animal if cells from such tumors have human chromosomes.

The terms "treating cancer", "cancer therapy", and the like mean generally a treatment that causes any improvement in a mammal having a cancer wherein the improvement is due to treatment with a peptide of the invention. The improvement can be either subjective or objective. For example, if the mammal is human, the patient may note improved vigor or vitality or decreased pain as subjective symptoms of improvement or response to therapy. Alternatively, the clinician may notice a decrease in tumor size or tumor burden based on physical exam, laboratory parameters, tumor markers, or radiographic findings.

The phrase "inhibiting tumor [or cell] growth" generally means that the rate of increase in mass, size, number and/or the metabolism of treated cells and/or tumors is slower as a result of treatment than that of nontreated cells and/or tumors. The growth of a cell line or tumor is said to be "inhibited" by a treatment if, when assayed by means such as radioisotope incorporation into the cells, the treated cells increase in number at a rate that is less than the proliferation rate of untreated control cells, and preferably less than about 50% of the untreated cell proliferation rate. More preferably, the growth rate is inhibited by at least 80%. If growth is assayed by a means such as plating in methylcellulose, the growth of a cell line is said to be "inhibited" if the treated cells give rise to less than the number of colonies that grow from a like number of untreated cells. Preferably, the number of colonies from treated cells is less than about 70% of the number from untreated cells. More preferably, the number of colonies is decreased by at least 50%. "Inhibition of cell growth" also encompasses zero growth and, most importantly, consequent death of the tumor cells and eradication of the tumor. When measured in vivo, "inhibition of tumor growth" encompasses fewer or smaller tumors (for example, smaller diameter) as compared to control animals or untreated patients. Progression of a tumor refers to events other than growth, such as morphological and physiological changes, and changes in gene and protein expression.

Inhibition can be evaluated by any accepted method of measuring whether growth or size of the tumor and/or increase in the number of cancerous or tumor cells has been slowed, stopped, or reversed. This includes direct observation and indirect evaluation such as subjective symptoms or objective signs. The clinician may notice a decrease in tumor size or tumor burden (number of tumors) based on physical exam, laboratory parameters, tumor markers, or radiographic findings. Alternatively, if the mammal is human, the patient may note improved vigor or vitality or decreased pain as subjective symptoms of improvement or response to therapy. Some laboratory signs that the clinician may observe for response to therapy include normalization of tests such as white blood cell count, red blood cell count, platelet count, erythrocyte sedimentation rate, and various enzyme levels such as transaminases and hydrogenases. Additionally, the clinician may observe a decrease in a detectable tumor marker such as prostatic specific antigen (PSA) or chorio embryonic antigen (CEA). Alternatively, other tests can be used to evaluate objective improvement such as sonograms, computerized axial tomography scans, nuclear magnetic resonance scans and positron emission testing.

The term "GPCR transmembrane peptide" can include a GPCR transmembrane domain fragment and/or a homologous peptide thereof, of at least 4–50, and preferably 4–30, and preferably at least 10–30 amino acids in length, such as 5, 6, 7, 8, 9, 10, 11, 12. 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 amino acids, or any range therein, or any corresponding sequences having conservative amino acid substitutions. Sample transmembrane peptides of the invention include, but are not limited to, the peptides listed in Table I of the present disclosure. A preferred transmembrane peptide of the present invention, when contacted with a cell or membrane structure (e.g., liposome) that contains a biologically active GPCR, modulates the biological activity of said GPCR in vitro, in vivo or in situ. The concentration of the peptide in a solution that contacts the cell in vivo (e.g, blood plasma or interstitial fluid) or in vitro (e.g., culture medium) is between 1 nanomolar and 50 micromolar, preferably between 1 nanomolar and 1 micromolar, and most preferably less than 5 micromolar.

The term "residue" refers to an amino acid or amino acid mimetic incorporated in a oligopeptide by an amide bond or amide bond mimetic.

"Negatively charged" refers to those amino acids, amino acid derivatives, amino acid mimetics and chemical moieties that are negatively charged at physiological pH. Negatively charged amino acids include, for example Asp and Glu. An "acidic" residue is a residue that is negatively charged at physiological pH.

"Positively charged" refers to those amino acids, amino acid derivatives, amino acid mimetics and chemical moieties that are positively charged at physiological pH. Positively charged amino acids include, for example, Lys and Arg. A "basic residue" is a residue that is positively charged at physiological pH.

"Neutral" refers to those amino acids, amino acid derivatives, amino acid mimetics and chemical moieties that are neither positively nor negatively charged at physiological pH.

"Consensus" sequence refers to peptides which are distinct from known GPCR sequences in critical structural features, but which are derived from consensus sequences of homologous GPCR transmembrane domains 1–7. Such consensus peptides may be derived by molecular modeling, optionally combined with hydrophobicity analysis and/or fitting to model helices, as non-limiting examples. Such modeling can be accomplished according to known method steps using known modeling algorithms, such as, but not limited to, ECEPP, INSIGHT, DISCOVER, CHEM-DRAW, AMBER, FRODO and CHEM-X. Such algorithms compare transmembrane domains between related G-protein coupled receptors, determine probable energy-minimized structures and define alternative consensus polypeptide fragments.

An amino acid or nucleic acid sequence of a GPCR polypeptide of the present invention is said to "substantially correspond" to another amino acid or nucleic acid sequence, respectively, if the sequence of amino acids or nucleic acid in both molecules provides polypeptides having biological activity that is substantially similar, qualitatively or quantitatively, to the corresponding fragment of at least one GPCR transmembrane domain, or which may be synergistic when

two or more transmembrane domains, consensus sequences or homologs thereof are present.

Additionally or alternatively, such "substantially corresponding" sequences of GPCR polypeptides include conservative amino acid or nucleotide substitutions, or degenerate nucleotide codon substitutions wherein individual amino acid or nucleotide substitutions are well known in the art.

The term "modulates a biological property or activity" means that in the presence of a test transmembrane peptide a measurable biological parameter or event is increased or decreased relative to a control in the absence of said peptide. Examples of biological property or activity include: the conformation of the GPCR, association of the GPCR with other molecules, signal transduction, extracellular secretion of cellular proteins, conformational changes in proteins, changes in enzymatic activity, changes in metabolic activity, changes in affinity for a ligand, changes in levels of viral infection, changes in vasodilation, modulation of heart rate, modulation of bronchodilation, modulation of endocrine secretions and modulation of gut peristalsis. Note that the GPCR biological activity need not be one that is limited to the precise in vivo role performed by the GPCR. The term also covers GPCR properties, such as viral protein binding, that are not part of the in vivo biological role of the GPCR. It further covers intrinsic properties of GPCRs that are only disclosed by experimental manipulation in the laboratory, such as the ability of GPCRs in artificial bilayers (e.g., liposomes) to interact with GPCR ligands.

"Signal transduction" is the process by which binding of a ligand to a receptor is translated into physiological change. In general, binding of a ligand to a receptor causes a change in a physical property of the receptor, for example a change in its conformation, or its orientation, or in its ability to bind other ligands. This change in a physical property can result, directly or indirectly, in increased or decreased ion fluxes, increased or decreased enzymatic activity, increased or decreased phosphorylation, increased or decreased translocation of the receptor or of any molecule (e.g., an inositol moiety or a G protein subunit) from one cellular compartment to another.

"GPCR ligands" refers to biological molecules that bind GPCRs in vitro, in situ or in vivo, and may include hormones, neurotransmitters, viruses or receptor binding domains thereof, G proteins, opsins, rhodopsins, nucleosides, nucleotides, coagulation cascade factors, odorants or pheromones, toxins, colony stimulating factors, platelet activating factors, neuroactive peptides, neurohumor, or any biologically active compounds, such as drugs or synthetic or naturally occurring compounds.

The phrase "inhibits HIV infection" means that a peptide of the invention inhibits binding of an HIV to a GPCR or inhibits a GPCR biological activity that mediates the entry and successful reproduction of an HIV virus into a GPCR-expressing cell.

The term "effective amount" means a dosage sufficient to produce a desired result. The desired result can be subjective or objective changes in the biological activity of a GPCR, especially signal transduction. Effective amounts of the GPCR polypeptide or composition, which may also include a functional derivative thereof, are from about 0.01 micrograms to about 100 mg/kg body weight, and preferably from about 10 micrograms to about 50 mg/kg body weight, such 0.05, 0.07, 0.09, 0.1, 0.5, 0.7, 0.9, 1, 2, 5, 10, 20, 25, 30, 40, 45, or 50 mg/kg.

A "conservative substitution", when describing a protein refers to a change in the amino acid composition of the protein that does not substantially alter the protein's activity. Thus, "conservatively modified variations" of a particular amino acid sequence refers to amino acid substitutions of those amino acids that are not critical for protein activity or substitution of amino acids with other amino acids having similar properties (e.g., acidic, basic, positively or negatively charged, polar or non-polar, etc.) such that the substitutions of even critical amino acids do not substantially alter activity. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such substitutions preferably are made in accordance with the following list, which substitutions may be determined by routine experimentation provide modified structural and functional properties of a synthesized polypeptide molecule, while maintaining the receptor binding, or inhibiting or mimicking biological activity, as determined by known GPCR receptor activity assays.

Original Residue	Exemplary Substitution
Ala	Gly; Ser
Arg	Lys
Asn	Gln; His
Asp	Glu
Cys	Se
Gln	Asn
Glu	Asp
Gay	Ala; Pro
His	Asn; Gln
Ile	Leu; Val
Leu	Ile; Val
Lys	Arg; Gln; Glu
Met	Leu; Tyr; Ile
Phe	Met; Leu; Tyr
Se	Thr
Thr	Se
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

Put differently, the following six groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

See also, Creighton (1984) PROTEINS, W.H. Freeman and Company. In addition, individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence are also "conservatively modified variations".

The term "substantial identity" or "substantial similarity" in the context of a polypeptide indicates that a polypeptides comprises a sequence which can have 40% sequence identity to a reference sequence, or preferably 70%, or more preferably 85% sequence identity to the reference sequence, or most preferably 90% identity over a comparison window of about 10–20 amino acid residues. "Percentage amino acid identity" or "percentage amino acid sequence identity" refers to a comparison of the amino acids of two polypeptides which, when optimally aligned, have approximately the designated percentage of the same amino acids. For example, "95% amino acid identity" refers to a comparison of the amino acids of two polypeptides which when opti-

mally aligned have 95% amino acid identity. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. Because the substituted amino acids have similar properties, the substitutions do not change the functional properties of the polypeptides. An indication that two polypeptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a polypeptide is substantially identical to a second polypeptide, for example, where the two peptides differ only by a conservative substitution. An indication that two nucleic acid sequences are substantially identical is that the polypeptide which the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid. Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48: 443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by visual inspection (see generally Ausubel et al., supra).

One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a tree or dendogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, *J. Mol. Evol.* 35: 351–360 (1987). The method used is similar to the method described by Higgins & Sharp, *CABIOS* 5:151–153 (1989). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. For example, a reference sequence can be compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps.

Another example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et al., *J. Mol. Biol.* 215:403410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al, supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=−4, and a comparison of both strands.

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873–5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

The term "nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides which have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g. degenerate codon substitutions) and complementary sequences and as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka et al., *J. Biol. Chem.* 260:2605–2608 (1985); and Cassol et al., 1992; Rossolini et al., *Mol. Cell. Probes* 8: 91–98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene.

The phrase "a nucleic acid sequence encoding" refers to a nucleic acid which contains sequence information for a structural RNA such as rRNA, a tRNA, or the primary amino acid sequence of a specific protein or peptide, or a binding site for a trans-acting regulatory agent. This phrase specifically encompasses degenerate codons (i.e., different codons which encode a single amino acid) of the native sequence or sequences which may be introduced to conform with codon preference in a specific host cell.

"Nucleic acid probes" may be DNA or RNA fragments. DNA fragments can be prepared, for example, by digesting plasmid DNA, or by use of PCR, or synthesized by either the phosphoramidite method described by Beaucage and Carruthers, *Tetrahedron Lett.* 22:1859–1862 (1981), or by the triester method according to Matteucci, et al., *J. Am. Chem. Soc.,* 103:3185 (1981), both incorporated herein by reference. A double stranded fragment may then be obtained, if desired, by annealing the chemically synthesized single strands together under appropriate conditions or by synthesizing the complementary strand using DNA polymerase with an appropriate primer sequence. Where a specific sequence for a nucleic acid probe is given, it is understood that the complementary strand is also identified and included. The complementary strand will work equally well in situations where the target is a double-stranded nucleic acid.

The terms "substantial identity" or "substantial sequence identity" as applied to nucleic acid sequences and as used herein and denote a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, and more preferably at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25–50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. The reference sequence may be a subset of a larger sequence.

The phrase "specifically binds to an antibody" or "specifically immunoreactive with", when referring to a protein or peptide, refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein and do not bind in a significant amount to other proteins present in the sample. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane (1988) ANTIBODIES, A LABORATORY MANUAL, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

DETAILED DESCRIPTION

All references cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the contents of the references cited within the references cited herein are also entirely incorporated by reference.

The present invention is based partly on evidence that transmembrane domains (TM) of GPCRs interact in a specific way in the assembly of receptor molecules. These interactions do not lead to a rigid structure, because some flexibility is required to allow for conformational changes to be made following ligand binding in order to provide the ability of the molecule to signal from the cell surface to the intracellular parts. It was also demonstrated for several GPCRs that the transmembrane domains are involved in ligand binding and thus contain openings that allow penetration of the ligands. Reports that expression of missing transmembrane domains rescues inactive truncated V2 vasopressin, beta-adrenergic and muscarinic M3 receptors (Schoneberg et al. EMBO J. 15: 1283 (1996); Wong et al., *J. Biol. Chem.* 265: 6219 (1990); Monnot et al., *J. Biol. Chem.* 271: 1507 (1996); Gudermann et al., *Annu. Rev. Neurosci.* 20: 399 (1997); Osuga et al., *J. Biol. Chem.* 272: 25006 (1997)) suggested peptide derived from the sixth transmembrane domain of P2-adrenergic receptor was found to inhibit receptor activation and dimerization (Hebert et al., *J. Biol. Chem.,* 271(27):16384–92 (1996)). All these observations suggested to us that targeting intramembrane interactions of GPCRs can specifically regulate GPCR function.

The hydrophobic nature of the transmembrane peptides makes their penetrations into the bilayer highly probable. Orientation inside the membrane can be controlled by addition of charged residues to the terminus that is supposed to be extracellular.

1. GPCR Peptides

GPCR polypeptides of the present invention, or nucleic acids encoding therefor, include a finite set of substantially corresponding sequences as substitution peptides or polynucleotide which can be routinely obtained by one of ordinary skill in the art, without undue experimentation, based on the teachings and guidance presented herein. For a detailed description of protein chemistry and structure, see Schulz et al., PRINCIPLES OF PROTEIN STRUCTURE, Springer-Verlag, New York, 1978, and Creighton, T. E., PROTEINS: STRUCTURE AND MOLECULAR PROPERTIES, W. H. Freeman & Co., San Francisco, 1983, which are hereby incorporated by reference. For a presentation of nucleotide sequence substitutions, such as codon preferences, see Ausubel et al, supra, at sections A.1.1–A.1.24, and Sambrook et al., supra, at Appendices C and D.

GPCR polypeptides include homologous sequences and/or fragments of at least one of transmembrane domain 1–7 of one or more GPCRs or homologs thereof, which GPCR polypeptides do not occur naturally, and/or which are provided in an isolated and/or purified form not found in nature.

However, in the context of the present invention, GPCR polypeptides of greater than 15–20 amino acids are preferred such that the GPCR polypeptides are able to span the lipid bilayer.

It is particularly preferred that peptides of the invention be selected or modified so that one end is charged and the other is neutral under physiological conditions. This is so that the peptide spontaneously inserts into a membrane. It is of particular importance that the peptide insert in the same orientation as the transmembrane GPCR domain from which it is derived.

Peptides of the invention can be derived from any of the 7 TM domains. Non-limiting, illustrative examples of GPCR TM1 and TM2 transmembrane domains that are used to generate molecules of the present invention include the following:

	TM1				SEQ ID NO:
	101			150	
GPCRAelegans	HPCEDIMGYV	WLTVVSFMVG	AVALVANLVV	ALVLLTSQ..	RRLNV 24
GRH	NLPTLTLSGK	IRVTVTFFLF	LLSATFNASF	LLKLQKWTQK	KEKGKKLSRM 25
TRH	RAVVALEYQV	VTILLVLIIC	GLGIVGNIMV	VLVVMR....	...TKHMRTP 26
FSHprec	NPCEDIMGYN	ILRVLIWFIS	ILAITGNIIV	LVILTTSQYK	.......LTV 27
TSHprec	NPCEDIMGYK	FLRIVVWFVS	LLALLGNVFV	LLILLTSHYK	.......LNV 28
LH_CGprec	NPCEDIMGYD	FLRVLIWLIN	ILAIMGNMTV	LFVLLTSRY.	......KLTV 29
PGE_EP1	PNTSAVPPSG	ASPALPIFSM	TLGAVSNLLA	LALLAQAA.G	RLRRRRSATT 30
PGE_EP2	SASLSPDRLN	SPVTIPAVMF	IFGVVGNLVA	IVVLCKS...	..RKEQKETT 31
PGE_EP3	QWLPPGE...	.SPAISSVMF	SAGVLGNLIA	LALLARRW.R	SAGRRSSLSL 32
PGF	SNTTCQTENR	LSVFFSVIFM	TVGILSNSLA	IAILMKAY.Q	RFRQKSKA.S 33
PGI	CRNLTYVRGS	VGPATSTLMF	VAGVVGNGLA	LGILSARRPA	RP......SA 34
TXA2	NITLEERRLI	ASPWFAASFC	VVGLASNLLA	LSVLAGA...	RQGGSHTRSS 35
PAF	HMDSEFRYTL	F.PIVYSIIF	VLGVIANGYV	LWVFARLY.P	CKKFNEIK.. 36
M2	...YKTFEVV	FIVLVAGSLS	LVTIIGNILV	MVSI.KVN.R	HLQT.....V 37
M4	HNRYETVEMV	FIATVTGSLS	LVTVVGNILV	MLSI.KVN.R	QLQT.....V 38
M1	..GKGPWQVA	FIGITTGLLS	LATVTGNLLV	LISF.KVN.T	ELKT.....V 39
M3	LGGHTVWQVV	FIAFLTGILA	LVTIIGNILV	IVSF.KVN.K	QLKT.....V 40
M5	LERHRLWEVI	TIAAVTAVVS	LITIVGNVLV	MISF.KVN.S	QLKT.....V 41
H1	KTTMASPQLM	PLVVVLSTIC	LVTVGLNLLV	LYAVRSER..	KLHT.....V 42
H2	FCLDSTACKI	TITVVLAVLI	LITVAGNVVV	CLAVGLNR..	RLRN.....L 43
5HT1A	ISDVTVSYQV	ITSLLLGTLI	FCAVLGNACV	VAAIALER..	SLQN.....V 44
5HT1B	QDSISLPWKV	LLVMLLALIT	LATTLSNAFV	IATVYRTR..	KLHT.....P 45
5HT1D	DPRTLQALKI	SLAVVLSVIT	LATVLSNAFV	LTTILLTR..	KLHT.....P 46
5HT1E	IRPKTITEKM	LICMTLVVIT	TLTTLLNLAV	IMAIGTTK..	KLHQ.....P 47
5HT1F	ELLNRMPSKI	LVSLTLSGLA	LMTTTINSLV	IAAIIVTR..	KLHH.....P 48
5HT2A	QEKN.....	WSALLTAVVI	ILTIAGNILV	IMAVSLEK..	KLQN.....A 49
5HT2B	IVEEQGNKLH	WAALLILMVI	IPTIGGNTLV	ILAVSLEK..	KLQY.....A 50
5HT2C	..QN.....	WPALSIVIII	IMTIGGNILV	IMAVSMEK..	KLHN.....A 51
5HT5A	SSPLLSVFGV	LILTLLGFLV	AATFAWNLLV	LATILRVR..	TFHR.....V 52
5HT5Brat	REPPFSAFTV	LVVTLLVLLI	AATFLWNLLV	LVTILRVR..	AFHR.....V 53
5HT6rat	GPPPAPGGSG	WVAAALCVVI	VLTAAANSLL	IVLICTQP..	AVRN.....T 54
5HT7	QINYGRVEKV	VIGSILTLIT	LLTIAGNCLV	VISVCFVK..	KLRQ.....P 55
alpha1A	GGLVVSAQGV	GVGVFLAAFI	LMAVAGNLLV	ILSVACNR..	HLQT.....V 56
alpha1B	..QLDITRAI	SVGLVLGAFI	LFAIVGNILV	ILSVACNR..	HLRT.....P 57
alpha1C	PAPVNISKAI	LLGVILGGLI	LFGVLCNILV	ILSVACHR..	HLHS.....V 58
alpha2A	...YSLQVTL	TLVCLAGLLM	LLTVFGNVLV	IIAVFTSR..	ALKA.....P 59
alpha2B	QDPYSVQATA	AIAAAITFLI	LFTIFGNALV	ILAVLTSR..	SLRA.....P 60

-continued

alpha2C1	RGQYSAGAVA GLAAVVGFLI VFTVVGNVLV VIAVLTSR.. ALRA.....P	61
alpha2C2	RGQYSAGAVA GLAAVVGFLI VFTVVGNVLV VIAVLTSR.. ALRA.....P	61
beta1	EPLSQQWTAG M.GLLMALIV LLIVAGNVLV IVAIAKTP.. RLQT.....L	62
beta2	QQRDEVWVVG M.GIVMSLIV LAIVFGNVLV ITAIAKFE.. RLQT.....V	63
beta3	GLPGVPWEAA LAGALLALAV LATVGGNLLV IVAIAWTP.. RLQT.....M	64
beta4turkey	SWAAVLSRQW AVGAALSITI LVIVAGNLLV IVAIAKTP.. RLQT.....M	65
D1A	VVERDFSVRI LTACFLSLLI LSTLLGNTLV CAAVIRFR.. HLRSK....V	66
D2	DGKADRPHYN YYATLLTLLI AVIVFGNVLV CMAVSREK.. ALQT.....T	67
D3	TGASQARPHA YYALSYCALI LAIVFGNGLV CMAVLKER.. ALQT.....T	68
D4	ASAGLAGQGA AALVGGVLLI GAVLAGNSLV CVSVATER.. ALQT.....P	69
D5	GAPPLGPSQV VTACLLTLLI IWTLLGNVLV CAAIVRSR.. HLRAN....M	70
A1	MPPSISAFQA AYIGIEVLIA LVSVPGNVLV IWAVKVNQ.. ALRD.....A	71
A2a	...MPIMGSS VYITVELAIA VLAILGNVLV CWAVWLNS.. NLQN.....V	72
A2b	..MLLETQDA LYVALELVIA ALSVAGNVLV CAAVGTAN.. TLQT.....P	73
A3	NSTTLSLANV TYITMEIFIG LCAIVGNVLV ICVVKLNP.. SLSQT....T	74
OCdrome	LAVPE.WEAL LTALVLSVII VLTIIGNILV ILSVFTYK.. PLRI.....V	75
ACTH	RNNSDCPRVV LPEEIFFTIS IVGVLENLIV LLAVFKNK.. NLQA.....P	76
MSH	QTGARCLEVS ISDGLFLSLG LVSLVENALV VATIAKNR.. NLHS.....P	77
MC3	SSSAFCEQVF IKPEIFLSLG IVSLLENILV ILAVVRNG.. NLHS.....P	78
MC4	SDGGCYEQLF VSPEVFVTLG VISLLENILV IVAIAKNK.. NLHS.....P	79
MC5	NKSSPCEDMG IAVEVFLTLG VISLLENILV IGAIVKNK.. NLHS.....P	80
melatonin	DGARPSWLAS ALACVLIFTI VVDILGNLLV ILSVYRNKKL ....RNA	81
oxytocin	R..RNEALAR VEVAVLCLIL LLALSGNACV LLALRTTRQK ....HSR	82
conopressinLs	FHGVDEDLLK IEIAVQATIL YMTLFGNGIV LLVLRLRRQK ....LRT	83
V1A	RDVRNEELAK LEIAVLAVTF AVAVLGNSSV LLAL..... .HRTPRKTSR	84
V1B	WLGRDEELAK VEIGVLATVL VLATGGNLAV LLTLGQLGRK ....RSR	85
V2	LDTRDPLLAR AELALLSIVF VAVALSNGLV LAALA..... RRGRRGHWAP	86
CCK_A	PRPSKEWQPA VQILLYSLIF LLSVLGNTLV ITVLI..... RNKRM..RTV	87
CCK_B	GAGTRELELA IRITLYAVIF LMSVGGNMLI IVVLGLS... ..RRL..RTV	88
NPY1	DCHLPLAMIF TLALAYGAVI ILGVSGNLAL IIIIL..... KQKEM..RNV	89
NTR	DVNTDIYSKV LVTAVYLALF VVGTVGNTVT AFTLAR.... KKSLQSLQST	90
NK1	QFVQPAWQIV LWAAAYTVIV VTSVVGNVVV MWIILA.... .HKRM..RTV	91
NK2	AFSMPSWQLA LWAPAYLALV LVAVTGNAIV IWIILA.... .HRRM..RTV	92
NK3	QFVQPSWRIA LWSLAYGVVV AVAVLGNLIV IWIILA.... .HKRM..RTV	93
blueops	YHIAPVWAFY LQAAFMGTVF LIGFPLNANV LVATL..... RYKKL..RQP	94
greenops	YHIAPRWVYH LTSVWMIFVV IASVFTNGLV LAATM..... KFKKL..RHP	95
redops	YHIAPRWVYH LTSVWMIFVV TASVFTNGLV LAATM..... KFKKL..RHP	96
rhodopsin	YYLAEPWQFS MLAAYMFLLI VLGFPINFLT LYVTVQ.... .HKKL..RTP	97
violetopsGg	YHIAPPWAFY LQTAFMGIVF AVGTPLNAVV LWVTVRYKRL ....RQP	98
opsin_crab	FPPMNPLWYS ILGVAMIILG IICVLGNGMV IYLMMTTKSL ....RTP	99

-continued

```
ET_Aprec QTKITSAFKY INTVISCTIF IVGMVGNATL LRIIYQ.... .NKCM..RNG 100
ET_Bprec PIEIKETFKY INTVVSCLVF VLGIIGNSTL LRIIYKNK.. ...CM..RNG 101
ET_Cfrog RAKIRHAFKY VTTILSCVIF LVGIVGNSTL LRIIYKNK.. ...CM..RNG 102
galanin PLFGIGVENF VTLVVFGLIF ALGVLGNSLV ITVLARSK.. ...PGKPRST 103
NMB GTTTELVIRC VIPSLYLLII TVGLLGNIML VKIFITNS.. ...AM..RSV 104
GRP DDWSHPGILY VIPAVYGVII LIGLIGNITL IKIFCTVK.. ...SM..RNV 105
BRS3 DNSPGIEALC AIYITYAVII SVGILGNAIL IKVFFKTK.. ...SM..QTV 106
deltaOP GSASSLALAI AITALYSAVC AVGLLGNVLV MFGIVRYT.. ...KM..KTA 107
kappaOP PAHISPAIPV IITAVYSVVF VVGLVGNSLV MFVIIRYT.. ...KM..KTA 108
muOP GSP.SMITAI TIMALYSIVC VVGLFGNFLV MYVIVRYT.. ...KM..KTA 109
OPRX GAFLPLGLKV TIVGLYLAVC VGGLLGNCLV MYVILRHT.. ...KM..KTA 110
CB1 FMVLNPSQQL AIAVLSLTLG TFTVLENLLV LCVILHSR.. SLRCR....P 111
CB2 YMILSGPQKT AVAVLCTLLG LLSALENVAV LYLILSSH.. QLRRK....P 112
SSTR1 TLSEGQGSAI LISFIYSVVC LVGLCGNSMV IYVILRYA.. ...KM..KTA 113
SSTR2 EPYYDLTSNA VLTFIYFVVC IIGLCGNTLV IYVILRYA.. ...KM..KTI 114
SSTR3 SPAGLAVSGV LIPLVYLVVC VVGLLGNSLV IYVVLRHT.. ...AS..PSV 115
SSTR4 GDARAAG.MV AIQCIYALVC LVGLVGNALV IFVILRYA.. ...KM..KTA 116
SSTR5 PAPSAGARAV LVPVLYLLVC AAGLGGNTLV IYVVLRFA.. ...KM..KTV 117
IL8A MLETETLNKY VVIIAYALVF LLSLLGNSLV MLVILYSR.. ...VG..RSV 118
IL8B EPESLEINKY FVVIIYALVF LLSLLGNSLV MLVILYSR.. ...VG..RSV 119
AT1a KAGRHNYIFV MIPTLYSIIF VVGIFGNSLV VIVIYFYM.. ...KL..KTV 120
AT1brat KAGRHNYIFV MIPTLYSIIF VVGIFGNSLV VIVIYFYM.. ...KL..KTV 120
AT2 QKPSDKH.LD AIPILYYIIF VIGFLVNIVV VTLFCCQK.. ...GP..KKV 121
BK1 APEAWDLLHR VLPTFIISIC FFGLLGNLFV LLVFLLPR.. .....RQLNV 122
BK2 QVEWLGWLNT IQPPFLWVLF VLATLENIFV LSVFCLHK.. .....SSCTV 123
P2Y7 PSLGVEFISL LAIILLSVAL AVGLPGNSFV VWSILKRMQ. ......KRSV 124
P2Y6 CVYREFDKRL LLPPVYSVVL VVGLPLNVCV IAQICASRR. ......TLTR 125
P2Y5 CSTEDSFKYT LYGCVFSMVF VLGLIANCVA IYIFTFTLK. ......VRNE 126
P2Y4 CWFDEDFKFI LLPVSYAVVF VLGLGLNAPT LWLFIFRLR. ......PWDA 127
P2Y3chick CTFHEEFKQV LLQLVYSVVF LLGLPLNAVV IGQIWLARK. ......ALTR 128
P2Y2 CFFNEDFKYV LLPVSYGVVC VLGLCLNAVG LYIFLCRLK. ......TWNA 129
P2Y1 ALTKTGFQFY YLPAVYILVF IIGFLGNSVA IWMFVFHMK. ......PWSG 130
THRprec GYLTSSWLTL FVPSVYTGVF VVSLPLNIMA IVVFILKM.. ...KV..KKP 131
C5a TSNTLRVPDI LALVIFAVVF LVGVLGNALV VWVTAFEA.. ...K...RTI 132
GP01mouse AESEPELVVN PWDIVLCSSG TLICCENAVV VLIIF.HSPS LR......AP 133
R334rat VESEPELVVN PWDIVLCSSG TLICCENAVV VLIIF.HSPS LR......AP 134
GP21mouse GPATLLPSPR AWDVVLCISG TLVSCENALV VAIIV.GTPA FR......AP 135
GCRCmouse AESQNPTVKA LLIVAYSFTI VFSLFGNVLV CHVIFK.NQR ......MHSA 136
TXKR ...QPPWAVA LWSLAYGAVV AVAVLGNLVV IWIVLA.HKR MR......TV 137
G10Drat MELNENTKQV VLFVFYLAIF VVGLVENVLV IC.VNCRRSG R......VGM 138
```

-continued

```
          RDC1 NMPNKSVLLY TLSFIYIFIF VIGMIANSVV VW.VNIQAKT TGYDT..... 139
          BLR1 ...MASFKAV FVPVAYSLIF LLGVIGNVLV LV.ILERHRQ TRSSTE.... 140
           CL5 REENANFNKI FLPTIYSIIF LTGIVGNGLV IL.VMGYQKK LRSMTDKYR. 141
          LCR1 REENANFNKI FLPTIYSIIF LTGIVGNGLV IL.VMGYQKK LRSMTDKYR. 141
          EBI1 KKDVRNFKAW FLPIMYSIIC FVGLLGNGLV VL.TYIYFKR LKTMTDTY.. 142
        RBS1rat LGDIVAFGTI FLSIFYSLVF TFGLVGNLLV VL.ALTNSRK SKSITDIY.. 143
          EBI2 LYAHHSTARI VMPLHYSLVF IIGLVGNLLA LV.VIVQNRK KINSTTLY.. 144
      GCRTchick CSTEDSFKYT LYGCVFSMVF VLGLIANCVA IY.IFTFTLK VRNETTTY.. 145
           APJ EYTDWKSSGA LIPAIYMLVF LLGTTGNGLV LWTVFRSSRE KRRSAD.... 146
         RTArat EQIATLPPPA VTNYIFLLLC LCGLVGNGLV LWFFGFSIK. .RT......P 147
         UHRrat SLQLVHQLKG LIVMLYSIVV VVGLVGNCLL VLVIARVR.. .....RLHNV 148
          FMRL1 EPAGHTVLWI FSLLVHGVTF VFGVLGNGLV IWVA.GFR.. .....MTRTV 149
          FMRL2 ESAGYTVLRI LPLVVLGVTF VLGVLGNGLV IWVA.GFR.. .....MTRTV 150
           fMLP VSAGYLFLDI ITYLVFAVTF VLGVLGNGLV IWVA.GFR.. .....MTHTV 151
    OLF1catfish NGFYNIPHTK YYYAFLCIAY AVTVLGNSFI MCTIYLAR.. .....SLHTA 152
    OLF3catfish TGLYNIPHAK YYYLFLCFVY TVTFLGNSFI MGTIYLAR.. .....SLHTA 153
    OLF8catfish GFHDLGEWGP ILSIPYLLMF LLSSTSNLTL IYLIISQR.. .....ALHSP 154
   OLF32Acatfish SGFSGIPFSQ YYFAFLIFIY IISLCGNSIV LFMILVDR.. .....TLHIP 155
   OLF32Bcatfish SGFSGIPFSQ YYFVFLIFIY IISLCGNSIV LFMILVDR.. .....TLHIP 156
   OLF32Ccatfish SGFSGIPFSQ YYFVFLIFIY IISLCGNSIV LFMILVDR.. .....TLHIP 156
   OLF32Dcatfish SGFSGIPFSQ YYFVFLIFIY IISLCGNSIV LFMILVDR.. .....TLHIP 156
    OLF47catfish IAYNSLGNKN YLILALGIIY LITLLCNFTL LAIILMNS.. .....SLQNP 157
   OLF202catfish FPGLPPNYYG LVSVVMFFVY VCTLIGNCTF FTLFLREK.. .....SLQKP 158
 OLFCOR1chicken LTD.NPGLQM PLFMVFLAIY TITLLTNLGL IALISVDL.. .....HLQTP 159
 OLFCOR2chicken LTD.NPRLQM PLFMVFLVIY TTTLLTNLGL IALIGMDL.. .....HLQTP 160
 OLFCOR3chicken LTD.NPGLQM PLFMVFLAIY TITLLTNLGL IRLISVDL.. .....HLQTP 161
 OLFCOR4chicken LTD.NPGLQM PLFMVFLAIY TITLLTNLGL IRLISVDL.. .....HLQTP 161
 OLFCOR5chicken LTD.NPRLQM PLFMVFLAIY TITLLANLGL IALISVDF.. .....HLQTP 162
 OLFCOR6chicken LTD.NPGLQM PLFMVFLAIY TITLLTNLGL IALIRIDL.. .....QLQTP 163
        OLFdog LPI.DPDQRD LFYALFLAMY VTTILGNLLI IVLIQLDS.. .....HLHTP 164
        OLF07E MSE.SPEQQQ ILFWMFLSMY LVTVVGNVLI ILAISSDS.. .....RLHTP 165
        OLF07I LPI.QPEQQN LCYALFLAMY LTTLLGNLLI IVLIRLDS.. .....HLHTP 166
        OLF07J FSS.FHEQQI TLFGVFLALY ILTLAGNIII VTIIRIDL.. .....HLHTP 167
   OLFOR3mouse VSD.HPHLEI IFFAVILASY LLTLVGNLTI ILLSRLDA.. .....RLHTP 168
        OLFrat LTK.QPELLL PLFFLFLVIY VLTVVGNLGM ILLIIVSP.. .....LLHTP 169
      OLFF3rat FVE.NKDLQP LIYGLFLSMY LVTVIGNISI IVAIISDP.. .....CLHTP 170
      OLFF5rat LSR.QPQQQQ LLFLLFLIMY LATVLGNLLI ILAIGTDS.. .....RLHTP 171
      OLFF6rat FPG.PRSMRI GLFLLFLVMY LLTVVGNLAI ISLVGAHR.. .....CLQTP 172
     OLFF12rat FTE.NPQLHF LIFALFLSMY LVTVLGNLLI IMAIITQS.. .....HLHTP 173
      OLFI3rat LPI.PEEHQH LFYALFLVMY LTTILGNLLI IVLVQLDS.. .....QLHTP 174
```

-continued

OLFI7rat	FPA.PAPLRV	LLFFLSLLXY	VLVLTENMLI	IIAIRNHP..	.....TLHKP 175
OLFI8rat	LPI.PPEHQQ	LFFALFLIMY	LTTFLGNLLI	VVLVQLDS..	.....HLHTP 176
OLFI9rat	LPF.PPEYQH	LFYALFLAMY	LTTLLGNLII	IILILLDS..	.....HLHTP 177
OLFI14rat	LPI.PSEYHL	LFYALFLAMY	LTIILGNLLI	IVLVRLDS..	.....HLHMP 178
OLFI15rat	LPI.PSEHQH	VFYALFLSMY	LTTVLGNLII	IILIHLDS..	.....HLHTP 179
OLFOR17_40	LLE.APGLQP	VVFVLFLFAY	LVTVRGNLSI	LAAVLVEP..	.....KLHTP 180
GUST27rat	....MILNCN	PFSGLFLSMY	LVTVLGNLLI	ILAVSSNSHL	.......HNL 181
RPE	PTGFGELEVL	AVGMVLLVEA	LSGLSLNTLT	IFSFCKTPEL	.......RTP 182
HHRF1	FTDVLNQSKP	VTLFLYGVVF	LFGSIGNFLV	IFTITWRRRI	.......QCS 183
HHRF2	NSTEIYQLFE	YTRLGVWLMC	IVGTFLNVLV	ITTILYYRRK	K......KSP 184
HHRF3	MTGPLFAIRT	TEAVLNTFII	FVGGPLNAIV	LITQLLTNRV	LG.....YST 185
MCP-1A	..DVKQIGAQ	LLPPLYSLVF	IFGFVGNMLV	VLILINCKKL	.......KCL 186
MCP-1B	..DVKQIGAQ	LLPPLYSLVF	IFGFVGNMLV	VLILINCKKL	.......KCL 186
PPR1bovine	..EVRKFAKV	FLPAFFTIAF	IIGLAGNSTV	VAIYAYYKKR	.......RTK 187

TM2

	151			200	SEQ ID NO:
GPCRAelegans	TRFLMCNLAF	ADFILGLYIF	ILTSVSAVTR	GDYHNYVQQW	QNGAGCKILG 188
GRH	.KLLLKHLTL	ANLLETLIVM	PLDGMWNITV	QWYA.....	.GELLCKVLS 189
TRH	TNCYLVSLAV	ADLMVLVAAG	LPNITDSIYG	SWVYGYV...	....GCLCIT 190
FSHprec	PRFLMCNLAF	ADLCIGIYLL	LIASVDIHTK	SQYHNYAIDW	QTGAGCDAAG 191
TSHprec	PRFLMCNLAF	ADFCMGMYLL	LIASVDLYTH	SEYYNHAIDW	QTGPGCNTAG 192
LH_CGprec	PRFLMCNLSF	ADFCMGLYLL	LIASVDSQTK	GQYYNHAIDW	QTGSGCSTAG 193
PGE_EP1	FLLFVASLLA	TDLAGHVIPG	ALVLRLYTA.	.......GRA	PAGGACHFLG 194
PGE_EP2	FYTLVCGLAV	TDLLGTLLVS	PVTIATYMKG	......QWPG	GQP.LCEYST 195
PGE_EP3	FHVLVTELVF	TDLLGTCLIS	PVVLASYARN	QT..LVALAP	ESR.ACTYFA 196
PGF	FLLLASGLVI	TDFFGHLING	AIAVFVYASD	KE..WIRFDQ	.SNVLCSIFG 197
PGI	FAVLVTGLAA	TDLLGTSFLS	PAVFVAYARN	SS..LLGLAR	GGPALCDAFA 198
TXA2	FLTFLCGLVL	TDFLGLLVTG	TIVVSQHAAL	FE..WHAVDP	GCR.LCRFMG 199
PAF	..IFMVNLTM	ADMLFLITLP	LWIVYYQ.NQ	GNWIL.....	PK.FLCNVAG 200
M2	NNYFLFSLAC	ADLIIGVFSM	NLYTLYTVIG	......YWPL	.GPVVCDLWL 201
M4	NNYFLFSLAC	ADLIIGAFSM	NLYTVYIIKG	......YWPL	.GAVVCDLWL 202
M1	NNYFLLSLAC	ADLIIGTFSM	NLYTTYLLMG	......HWAL	.GTLACDLWL 203
M3	NNYFLLSLAC	ADLIIGVISM	NLFTTYIIMN	......RWAL	.GNLACDLWL 204
M5	NNYYLLSLAC	ADLIIGIFSM	NLYTTYILMG	......RWAL	.GSLACDLWL 205
H1	GNLYIVSLSV	ADLIVGAVVM	PMNILYLLMS	......KWSL	.GRPLCLFWL 206
H2	TNCFIVSLAI	TDLLLGLLVL	PFSAIYQLSC	......KWSF	G.KVFCNIYT 207
5HT1A	ANYLIGSLAV	TDLMVSVLVL	PMAALYQVLN	......KWTL	.GQVTCDLFI 208
5HT1B	ANYLIASLAV	TDLLVSILVM	PISTMYTVTG	......RWTL	.GQVVCDFWL 209
5HT1D	ANYLIGSLAT	TDLLVSILVM	PISIAYTITH	......TWNF	.GQILCDIWL 210

-continued

5HT1E	ANYLICSLAV	TDLLVAVLVM	PLSIIYIVMD	......RWKL	.GYFLCEVWL 211
5HT1F	ANYLICSLAV	TDFLVAVLVM	PFSIVYIVRE	......SWIM	.GQVVCDIWL 212
5HT2A	TNYFLMSLAI	ADMLLGFLVM	PVSMLTILYG	.....YRWPL	P.SKLCAVWI 213
5HT2B	TNYFLMSLAV	ADLLVGLFVM	PIALLTIMFE	.....AMWPL	P.LVLCPAWL 214
5HT2C	TNYFLMSLAI	ADMLVGLLVM	PLSLLAILYD	.....YVWPL	P.RYLCPVWI 215
5HT5A	PHNLVASMAV	SDVLVAALVM	PLSLVHELS.	....GRRWQL	.GRRLCQLWI 216
5HT5Brat	PHNLVASTAV	SDVLVAALVM	PLSLVSELSA	....GRRWQL	.GRSLCHVWI 217
5HT6rat	SNFFLVSLFT	SDLMVGLVVM	PPAMLNALYG	......RWVL	A.RGLCLLWT 218
5HT7	SNYLIVSLAL	ADLSVAVAVM	PFVSVTDLIG	G.....KWIF	.GHFFCNVFI 219
alpha1A	TNYFIVNLAV	ADLLLSATVL	PFSATMEVLG	......FWAF	G.RAFCDVWA 220
alpha1B	TNYFIVNLAM	ADLLLSFTVL	PFSAALEVLG	......YWVL	G.RIFCDIWA 221
alpha1C	THYYIVNLAV	ADLLLTSTVL	PFSAIFEVLG	......YWAF	G.RVFCNIWA 222
alpha2A	QNLFLVSLAS	ADILVATLVI	PFSLANEVMG	.Y.....WYF	.GKAWCEIYL 223
alpha2B	QNLFLVSLAA	ADILVATLII	PFSLANELLG	.Y.....WYF	R.RTWCEVYL 224
alpha2C1	QNLFLVSLAS	ADILVATLVM	PFSLANELMA	.Y.....WYF	.GQVWCGVYL 225
alpha2C2	QNLFLVSLAS	ADILVATLVM	PFSLANELMA	.Y.....WYF	.GQVWCGVYL 225
beta1	TNLFIMSLAS	ADLVMGLLVV	PFGATIVVWG	......RWEY	GS.FFCELWT 226
beta2	TNYFITSLAC	ADLVMGLAVV	PFGAAHILMK	......MWTF	GN.FWCEFWT 227
beta3	TNVFVTSLAA	ADLVMGLLVV	PPAATLALTG	......HWPL	GA.TGCELWT 228
beta4turkey	TNVFVTSLAC	ADLVMGLLVV	PPGATILLSG	......HWPY	GT.VVCELWT 229
D1A	TNFFVISLAV	SDLLVAVLVM	PWKAVAEIAG	......FWPF	GS..FCNIWV 230
D2	TNYLIVSLAV	ADLLVATLVM	PWVVYLEVVG	E......WKF	S.RIHCDIFV 231
D3	TNYLVVSLAV	ADLLVATLVM	PWVVYLEVTG	GV.....WNF	S.RICCDVFV 232
D4	TNSFIVSLAA	ADLLLALLVL	PLFVYSEVQG	GA.....WLL	SPRLC.DALM 233
D5	TNVFIVSLAV	SDLFVALLVM	PWKAVAEVAG	......YWPF	GA..FCDVWV 234
A1	TFCFIVSLAV	ADVAVGALVI	PLAILINIGP	QTYFHTCL..	.......MVA 235
A2a	TNYFVVSLAA	ADIAVGVLAI	PFAITISTGF	CAACHGCL..	.......FIA 236
A2b	TNYFLVSLAA	ADVAVGLFAI	PFAITISLGF	CTDFYGCL..	.......FLA 237
A3	TFYFIVSLAL	ADIAVGVLVM	PLAIVVSLGI	TIHFYSCL..	.......FMT 238
OCdrome	QNFFIVSLAV	ADLTVALLVL	PFNVAYSILG	R......WEF	GI.HLCKLWL 239
ACTH	MYFFICSLAI	SDMLGSLYKI	LENILIILRN	MGYLKPRGSF	ET.TADDIID 240
MSH	MYCFICCLAL	SDLLVSGTNV	LETAVILLLE	AGALVARAAV	LQ.QLDNVID 241
MC3	MYFFLCSLAV	ADMLVSVSNA	LETIMIAIVH	SDDYTFEDQF	IQ.HMDNIFD 242
MC4	MYFFICSLAV	ADMLVSVSNG	SETIIITLLN	STD.TDAQSF	TV.NIDNVID 243
MC5	MYFFVCSLAV	ADMLVSMSSA	WETITIYLLN	NKHLVIADAF	V.RHIDVNFD 244
melatonin	GNIFVVSLAV	ADLVVAIYPY	PLVLMSIFNN	GWNLGYLH..	.....CQVSG 245
oxytocin	LFFFMKHLSI	ADLVVAVFQV	LPQLLWDITF	RFYGP.....	..DLLCRLVK 246
conopressinLs	MQWFIAHLAF	ADIFVGFFNI	LPQLISDVTI	VFHGDD....	...FTCRFIK 247
V1A	MHLFIRHLSL	ADLAVAFFQV	LPQMCWDITY	RFRGPD....	...WLCRVVK 248
V1B	MHLFVLHLAL	TDLAVALFQV	LPQLLWDITY	RFQGP.....	..DLLCRAVK 249

-continued

V2	IHVFIGHLCL	ADLAVALFQV	LPQLAWKATD	RFRGPD....	...ALCRAVK 250
CCK_A	TNIFLLSLAV	SDLMLCLFCM	PFNLIPNLLK	DFIFGS....	...AVCKTTT 251
CCK_B	TNAFLLSLAV	SDLLLAVACM	PFTLLPNLMG	TFIFGT....	...VICKAVS 252
NPY1	TNILIVNLSF	SDLLVAIMCL	PFTFVYTLMD	HWVFGE....	...AMCKLNP 253
NTR	VHYHLGSLAL	SDLLTLLLAM	PVELYNFIWV	HHPWAF....	.GDAGCRGYY 254
NK1	TNYFLVNLAF	AEASMAAFNT	VVNFTYAVHN	EWYYGL....	...FYCKFHN 255
NK2	TNYFIVNLAL	ADLCMAAFNA	AFNFVYASHN	IWYFGR....	...AFCYFQN 256
NK3	TNYFLVNLAF	SDASMAAFNT	LVNFIYALHS	EWYFGA....	...NYCRFQN 257
blueops	LNYILVNVSF	GGFLLCIFSV	FPVFVASCNG	YFVFGR....	...HVCALEG 258
greenops	LNWILVNLAV	ADLAETVIAS	TISVVNQVYG	YFVLGH....	...PMCVLEG 259
redops	LNWILVNLAV	ADLAETVIAS	TISIVNQVSG	YFVLGH....	...PMCVLEG 260
rhodopsin	LNYILLNLAV	ADLFMVLGGF	TSTLYTSLHG	YFVFGP....	...TGCNLEG 261
violetopsGg	LNYILVNISA	SGFVSCVLSV	FVVFVASARG	YFVFG.....	..KRVCELEA 262
opsin_crab	TNLLVVNLAF	SDFCMMAFMM	PTMTSNCFAE	TWILG.....	..PFMCEVYG 263
ETA_prec	PNALIASLAL	GDLIYVVIDL	PINVFKLLAG	RWPFDH.NDF	GV.FLCKLFP 264
ETB_prec	PNILIASLAL	GDLLHIVIDI	PINVYKLLAE	DWPFGAE...	....MCKLVP 265
ET_Cfrog	PNVLIASLAL	GDLFYILIAI	PIISISFWLS	TGH.....	....SEYIYQ 266
galanin	TNLFILNLSI	ADLAYLLFCI	PFQATVYALP	TWVLGA....	...FICKFIH 267
NMB	PNIFISNLAA	GDLLLLLTCV	PVDASRYFFD	EWMFGKVG..	.....CKLIP 268
GRP	PNLFISSLAL	GDLLLLITCA	PVDASRYLAD	RWLFGRIG..	.....CKLIP 269
BRS3	PNIFITSLAF	GDLLLLLTCV	PVDATHYLAE	GWLFGRIG..	.....CKVLS 270
deltaOP	TNIYIFNLAL	ADALATSTLP	FQSAKYLMET	.WPFGE....	...LLCKAVL 271
kappaOP	TNIYIFNLAL	ADALVTTTMP	FQSTVYLMNS	.WPFGD....	...VLCKIVI 272
muOP	TNIYIFNLAL	ADALATSTLP	FQSVNYLMGT	.WPFGT....	...ILCKIVI 273
OPRX	TNIYIFNLAL	ADTLVLLTLP	FQGTDILLGF	.WPFGN....	...ALCKTVI 274
CB1	SYHFIGSLAV	ADLLGSVIFV	YSFIDFHVFH	RKD.....	.SRNVFLFKL 275
CB2	SYLFIGSLAG	ADFLASVVFA	CSFVNFHVFH	GVD.....	.SKAVFLLKI 276
SSTR1	TNIYILNLAI	ADELLMLSVP	FLVTSTLLRH	.WPFGA....	...LLCRLVL 277
SSTR2	TNIYILNLAI	ADELFMLGLP	FLAMQVALVH	.WPFGK....	...AICRVVM 278
SSTR3	TNVYILNLAL	ADELFMLGLP	FLAAQNALSY	.WPFGS....	...LMCRLVM 279
SSTR4	TNIYLLNLAV	ADELFMLSVP	FVASSAALRH	.WPFGS....	...VLCRAVL 280
SSTR5	TNIYILNLAV	ADVLYMLGLP	FLATQNAASF	.WPFGP....	...VLCRLVM 281
IL8A	TDVYLLNLAL	ADLLFALTLP	IWAA..SKVN	GWIFGT....	...FLCKVVS 282
IL8B	TDVYLLNLAL	ADLLFALTLP	IWAA..SKVN	GWIFGT....	...FLCKVVS 282
AT1a	ASVFLLNLAL	ADLCFLLTLP	LWAVYTAMEY	RWPFGN....	...YLCKIAS 283
AT1brat	ASVFLLNLAL	ADLCFLLTLP	LWAVYTAMEY	RWPFGN....	...HLCKIAS 284
AT2	SSIYIFNLAV	ADLLLLATLP	LWATYYSYRY	DWLFGP....	...VMCKVFG 285
BK1	AEIYLANLAA	SDLVFVLGLP	FWAENIWNQF	NWPFGA....	...LLCRVIN 286
BK2	AEIYLGNLAA	ADLILACGLP	FWAITISNNF	DWLFGE....	...TLCRVVN 287
P2Y7	TALMVLNLAL	ADLAVLLTAP	FFLHFLAQGT	WSFGLA....	....GCRLCH 288

-continued

```
          P2Y6 SAVYTLNLAL ADLLYACSLP LLIYNYARGD HWPFGD.... ...LACRLVR 289
          P2Y5 TTTYMLNLAI SDLLFVFTLP FRIYYFVVRN .WPFGD.... ...VLCKISV 290
          P2Y4 TATYMFHLAL SDTLYVVSLP TLIYYYAAHN HWPFGT.... ...EICKFVR 291
      P2Y3chick TTIYMLNLAM ADLLYVCSLP LLIYNYTQKD YWPFGD.... ...FTCKFVR 292
          P2Y2 STTYMFHLAV SDALYAASLP LLVYYYARGD HWPFST.... ...VLCKLVR 293
          P2Y1 ISVYMFNLAL ADFLYVLTLP ADIFYYFNKT DWIFGD.... ...AWCKLQR 294
        THRprec AVVYMLHLAT ADVLFVSVLP FKISYYFSGS DWQFGS.... ...ELCRFVT 295
            C5a NAIWFLNLAV ADFLSCLALP ILFTSIVQHH WPFGGA.... ... ACSILP 296
      GP01mouse MFLLIGSLAL ADLLAGLGLI INFVFAYLLQ ....SE.... ...ATKLVTI 297
        R334rat MFLLIGSLAL ADLLAGLGLI INFVFAYLLQ ....SE.... ...ATKLVTI 297
      GP21mouse MFLLVGSLAV ADLLAGLGLV LHFAADFCIG ....SP.... ...EMSLMLV 298
      GCRCmouse TSLFIVNLAV ADIMITLLNT PFTLVRFVNS TWVFGK.... ...GMCHVSR 299
           TXKR TNSFLVNLAF ADAAMAALNA LVNFIYALHG EWYFGA.... ...NYCRFQN 300
        G10Drat LNLYILNMAV ADLGIILSLP VWMLEVMLEY TWLWGS.... ...FSCRFIH 301
           RDC1 .HCYILNLAI ADLWVVLTIP VWVVSLVQHN QWPMGE.... ...LTCKVTH 302
           BLR1 ..TFLFHLAV ADLLLVFILP FAVAEGSV.. GWVLGT.... ...FLCKTVI 303
             CL5 .....LHLSV ADLLFVITLP FWAVDAVA.. NWYFGN.... ...FLCKAVH 304
           LCR1 .....LHLSV ADLLFVITLP FWAVDAVA.. NWYFGN.... ...FLCKAVH 304
           EBI1 ....LLNLAV ADILFLLTLP FWAYSAAK.. SWVFGV.... ...HFCKLIF 305
        RBS1rat ....LLNLAL SDLLFVATLP FWTHYLIS.. HEGLHN.... ...AMCKLTT 306
           EBI2 ....STNLVI SDILFTTALP TRIAYYAMGF DWRIGD.... ...ALCRITA 307
      GCRTchick ....MLNLAI SDLLFVFTLP FRIYYFVVR. NWPFGD.... ...VLCKISV 308
            APJ ..IFIASLAV ADLTFVVTLP LWATYTYRDY DWPFGT.... ...FFCKLSS 309
          RTArat FSIYFLHLAS ADGIYLFSKA VIALLNMGTF LGSFPD.... ...YVRRVSR 310
          UHRrat TNFLIGNLAL SDVLMCAACV PLTLAYAFEP RGWVFG.... ..GGLCHLVF 311
           FMRL1 NTICYLNLAL ADFSFSAILP FRMVSVAMRE KWPFAS.... ...FLCKLVH 312
           FMRL2 TTICYLNLAL ADFSFTATLP FLIVSMAMGE KWPFGW.... ...FLCKLIH 313
            fMLP TTISYLNLAV ADFCFTSTLP FFMVRKAMGG HWPFGW.... ...FLCKFLF 314
    OLF1catfish KYITVFNLAL SDLGGSSALI PKLIDTFLF. ......ENQV ISYEACLANM 315
    OLF3catfish KYIAVFNLAL SDLCGSSALI PKLLDMLLF. ......ENQS ISYEACLSNM 316
    OLF8catfish MCILIGLMAV VDLSMPIFCV PNMLLSFLF. ......NWKG ISLVGCLVQM 317
   OLF32Acatfish KYMGIFNLAL SDFGETNVLI PSLVKTLFF. ......DSQY ISYDACLANM 318
   OLF32Bcatfish KYMGIFNLAL SDFGETNALI PSLVKTLFF. ......DSQY ISYDACLANM 319
   OLF32Ccatfish KYMGIFNLAL SDIGETNALI PSLVKTLFF. ......DSQY ISYDACLTNM 320
   OLF32Dcatfish KYMGIFNLAL SDFGETNALI PSLVKTLFF. ......DSQY ISYDACLANM 319
    OLF47catfish KFLAVFNLAV VDISINSVII PQMVPVFVF. ......NLNH ISFESCFSQM 321
   OLF202catfish MYYIMLNLAA SDVLFSTTTL PKIIARYWF. ......GDGS ISFVGCFIQM 322
OLFCOR1chicken MYIFLQNLSF TDAAYSTVIT PKMLATFL.. .....EERKT ISYVGCILQY 323
OLFCOR2chicken MYIFLQNLSF TDAAYSTVIT PKMLATFL.. .....EERRT ISYVGCILQY 324
OLFCOR3chicken MYIFLQNLSF TDAAYSTVIT PKMLATFL.. .....EERKT ISYVGCILQY 323
```

-continued

OLFCOR4chicken	MYIFLQNLSF	TDAAYSTVIT	PKMLATFL..	.....EERKT ISYVGCILQY 323
OLFCOR5chicken	MYIFLQNLSF	TDAAYSTVIT	PKMLATFL..	.....EERRT ISYVGCILQY 324
OLFCOR6chicken	MYIFLQNLSF	TDAVYSTVIT	PKMLATFL..	.....EETKT ISYVGCILQY 325
OLFdog	MYLFLSNLSF	SDLCFSSVTM	PKLLQNMQ..	.....SQVPS IPYAGCLTQM 326
OLF07E	VYFFLANLSF	TDLFFVTNTI	PKMLVNLQ..	.....SHNKA ISYAGCLTQL 327
OLF07I	MYLFLSNLSF	SDLCFSSVTI	PKLLQNMQ..	.....NQDPS IPYADCLTQM 328
OLF07J	MYFFLSMLST	SETVYTLVIL	PRMLSSLV..	.....GMSQP MSLAGCATQM 329
OLFOR3mouse	MYFFLSNLSS	LDLAFTTSSV	PQMLKNLW..	.....GPDKT ISYGGCVTQL 330
OLFrat	MYYFLSSLSF	VDLCYSTVIT	PKMLVNFL..	.....GKKNF ITYSECMAQF 331
OLFF3rat	MYFFLSNLSF	VDICFISTTV	PKMLVNIQ..	.....TQNNV ITYAGCITQI 332
OLFF5rat	MYFFLSNLSF	VDVCFSSTTV	PKVLANHI..	.....LGSQA ISFSGCLTQL 333
OLFF6rat	MYFFLCNLSF	LEIWFTTACV	PKTLATF...	....APRGGV ISLAGCATQM 334
OLFF12rat	MYFFLANLSF	VDTCFTSTTI	PKMLVNIY..	.....TQSKS ITYEDCISQM 335
OLFI3rat	MYLFLSNLSF	SDLCFSSVTM	PKLLQNMR..	.....SQDTS IPYGGCLAQT 336
QLFI7rat	MYFFLANMSF	LEIWYVTVTI	PKMLAGFIG.	..SKENHGQL ISFEACMTQL 337
OLFI8rat	MYLFLSNLSF	SDLCFSSVTM	LKLLQNIQ..	.....SQVPS ISYAGCLTQI 338
OLFI9rat	MYLFLSNLSF	ADLCFSSVTM	PKLLQNMQ..	.....SQVPS IPYAGCLAQI 339
OLFI14rat	MYLFLSNLSF	SDLCFSSVTM	PKLLQNMQ..	.....SQVPS ISYTGCLTQL 340
OLFI15rat	MYLFLSNLSF	SDLCFSSVTM	PKLLQNMQ..	.....SQVPS IPFAGCLTQL 341
OLFOR17_40	MYFFLGNLSV	LDVGCISVTV	PSMLSRLL..	.....SRKRA VPCGACLTQL 342
GUST27rat	MYFFLSNLSF	VDICFISTTI	PKMLVNIH..	.....SQTKD ISYIECLSQV 343
RPE	CHLLVLSLAL	ADSGISLNAL	VAATSSLLRR	WPYG..... ..SDGCQAHG 344
HHRF1	GDVYFINLAA	ADLLFVCTLP	LWMQYLLDHN	SLA..... ..SVPCTLLT 345
HHRF2	SDTYICNLAV	ADLLIVVGLP	FFLEYAKHHP	KLSR..... ..EVVCSGLN 346
HHRF3	PTIYMTNLYS	TNFLTLTVLP	FIVLSNQWLL	PAG..... ..VASCKFLS 347
MCP-1A	TDIYLLNLAI	SDLLFLITLP	LWAHSAANEW	VFG..... ..NAMCKLFT 348
MCP-1B	TDIYLLNLAI	SDLLFLITLP	LWAHSAANEW	VFG..... ..NAMCKLFT 348
PPR1bovine	TDVYILNLAV	ADLFLLFTLP	FWAVNAVHGW	VLG..... ..KIMCKVTS 349

The above sequences were obtained from a public database. Examples of TM3 and TM5 transmembrane domain sequences are included in WO 94/05695, and are incorporated by reference. Examples of TM4, TM6, and TM7 transmembrane domain sequences can similarly be obtained from public sources.

2. Synthesis of Peptides

The peptides or fragments of GPCRs may be isolated from a natural source, chemically synthesized or produced recombinantly, in order to provide GPCR polypeptides which mimic, modulate or inhibit binding of ligands to G-protein coupled receptors.

a. Chemical Synthesis of GPCR Transmembrane Peptides

Transmembrane peptides of the present invention are be made using well known peptide synthesis procedures, as described in e.g., Merrifield, Science 232: 341–347 (1986), Barany and Merrifield, THE PEPTIDES, Gross and Meienhofer, eds. (N.Y., Academic Press), pp. 1–284 (1979); and Stewart and Young, SOLID PHASE PEPTIDE SYNTHESIS (Rockford, Ill., Pierce), 2d Ed. (1984), incorporated by reference herein.

The peptides were synthesized by a flow-through solid phase peptide synthesis on 432A Applied Biosystems Peptide Synthesizer utilizing Fmoc amino acid derivatives. To overcome aggregation that frequently occurs during the synthesis of hydrophobic peptides and leads to the blockage of the growing peptide chain, FmocHmb derivatives of Ala, Val and Leu were introduced into the different sequences, but not more than two derivatives of that type per peptide to prevent sterical hindrance during the synthesis. Coupling on the step after FmocHmb amino acid was prolonged to 90 min, since this protection group causes slowing of the next coupling step due to steric hindrance (T. Johnson, M. Quibell, *Tetrahedron Lett.* 35:463 (1994). The purity of the peptides was assessed by reverse phase HPLC and the structures were confirmed by matrix-assisted laser-desorption mass spectrometry.

b. Recombinant Production of GPCR Transmembrane Peptides

Nucleic acids that encode GPCR transmembrane peptides may be obtained by synthesizing, isolating or obtaining a nucleic acid sequence that encodes a GPCR protein, and subcloning a region of the sequence that encodes a desired transmembrane peptide.

i. Chemical Synthesis of Oligonucleotides

Oligonucleotides used in the present invention, including sequences that encode transmembrane peptides, are optionally chemically synthesized using the solid phase phosphoramidite triester method of Beaucage and Carruthers, *Tetrahedron Lett.,* 22(20): 1859–1862 (1981) using an automated synthesizer as described in Needham-VanDevanter et al., *Nucleic Acids Res.,* 12: 6159–6168 (1984). The chemically synthesized oligonucleotides are then purified by native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson and Regnier, *J. Chrom.,* 255: 137–149 (1983). The sequence of the synthetic oligonucleotide is verified, for example by using the chemical degradation method of Maxam and Gilbert in Grossman, L. and Moldave, D., eds. Academic Press, New York, *Methods in Enzymology,* 65:499–560 (1980).

The DNA sequences of the present invention coding for GPCR transmembrane peptides protein can be modified (i.e., mutated) to prepare various mutations. Such mutations may be either degenerate, i.e., the mutation does not change the amino acid sequence encoded by the mutated codon, or non-degenerate, i.e., the mutation changes the amino acid sequence encoded by the mutated codon. These modified DNA sequences may be prepared, for example, by mutating known sequences so that the mutation results in the deletion, substitution, insertion, inversion or addition of one or more amino acids in the encoded polypeptide using various methods known in the art. For example, the methods of site-directed mutagenesis described in Taylor et al., Nucl. Acids Res. 13, 8749–8764 (1985) and Kunkel, *Proc. Natl. Acad. Sci. USA* 82, 482–492 (1985) may be employed. In addition, kits for site-directed mutagenesis may be purchased from commercial vendors. For example, a kit for performing site-directed mutagenesis may be purchased from Amersham Corp. (Arlington Heights, Ill.). Both degenerate and non-degenerate mutations may be advantageous in producing or using the polypeptides of the present invention. For example, these mutations may permit higher levels of production, easier purification, or provide additional restriction endonuclease-recognition sites. All such modified DNAs (and the encoded polypeptide molecules) are included within the scope of the present invention.

ii. Recombinant Isolation of GPCR Transmembrane Peptide-Encoding Nucleic Acids

Nucleic acids that encode GPCR can be isolated from genomic or cDNA libraries, subcloning the library into expression vectors, labelling probes, DNA hybridization, and the like, as described in Sambrook, et al., Molecular Cloning—A Laboratory Manual (2nd Ed.), Vol. 1–3, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989. This manual is hereinafter referred to as "Sambrook, et al.", and is incorporated herein by reference.

Various methods of amplifying target sequences, such as the polymerase chain reaction (PCR), can also be used to prepare DNA encoding GPCR transmembrane peptides or a peptide fragment thereof. In PCR techniques, oligonucleotide primers complementary to the two 3' borders of the DNA region to be amplified are synthesized. The polymerase chain reaction is then carried out using the two primers. See PCR Protocols: A Guide to Methods and Applications. (Innis, M, Gelfand, D., Sninsky, J. and White, T., eds.), Academic Press, San Diego (1990). Primers can be selected to amplify the entire regions encoding a full-length GPCR transmembrane peptides or to amplify smaller DNA segments as desired. Once selected sequences are PCR-amplified, oligonucleotide probes can be prepared from sequence obtained. These probes can then be used to isolate DNA's encoding GPCR transmembrane peptides or a peptide fragment thereof.

iii. Recombinant Expression of Transmembrane Peptide-encoding Nucleic Acids

Once a nucleic acid encoding a GPCR transmembrane peptides or a peptide fragment thereof is isolated and cloned, the nucleic acid is expressed in a variety of recombinantly engineered cells to ascertain that the isolated nucleic acid indeed encodes the desired GPCR transmembrane peptides or a peptide fragment thereof. The expression of natural or synthetic nucleic acids is typically achieved by operably linking a nucleic acid of interest to a promoter (which is either constitutive or inducible), incorporating the construct into an expression vector, and introducing the vector into a suitable host cell. Typical vectors contain transcription and translation terminators, transcription and translation initiation sequences, and promoters useful for regulation of the expression of the particular nucleic acid. The vectors optionally comprise generic expression cassettes containing at least one independent terminator sequence, sequences permitting replication of the cassette in eukaryotes, or prokaryotes, or both, (e.g., shuttle vectors) and selection markers for both prokaryotic and eukaryotic systems. Vectors are suitable for replication and integration in prokaryotes, eukaryotes, or preferably both. See, Giliman and Smith (1979), *Gene,* 8: 81–97; Roberts et al. (1987), *Nature,* 328:731–734; Berger and Kimmel, Guide to Molecular Cloning Techniques, *Methods in Enzymology* 152, Academic Press, Inc., San Diego, Calif. (Berger); Sambrook et al. (1989), Molecular Cloning—A Laboratory Manual (2nd ed.) Vol. 1–3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, N.Y., (Sambrook); and F. M. Ausubel et al., Current Protocols in Molecular Biology, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1994 Supplement) (Ausubel). Product information from manufacturers of biological reagents and experimental equipment also provide information useful in known biological methods. Such manufacturers include the SIGMA chemical company (Saint Louis, Mo.), R&D systems (Minneapolis, Minn.), Pharmacia LKB Biotechnology (Piscataway, N.J.), CLONTECH Laboratories, Inc. (Palo Alto, Calif.), Chem. Genes Corp., Aldrich Chemical Company (Milwaukee, Wis.), Glen Research, Inc., GIBCO BRL Life Technologies, Inc. (Gaithersberg, Md.), Fluka Chemica-Biochemika Analytika (Fluka Chemie AG, Buchs, Switzerland), and Applied Biosystems (Foster City, Calif.), as well as many other commercial sources known to one of skill.

The nucleic acids (e.g., coding sequences, promoters and vectors) used in the present method can be isolated from natural sources, obtained from such sources as ATCC or GenBank libraries, or prepared by synthetic methods. Synthetic nucleic acids can be prepared by a variety of solution or solid phase methods. Detailed descriptions of the procedures for solid phase synthesis of nucleic acids by phosphite-triester, phosphotriester, and H-phosphonate chemistries are widely available. See, for example, Itakura, U.S. Pat. No. 4,401,796; Caruthers, et al., U.S. Pat. Nos. 4,458,066 and 4,500,707; Beaucage, et al., (1981) *Tetrahedron Lett.,* 22:1859–1862; Matteucci, (1981) et al., *J. Am. Chem.*

Soc., 103:3185–3191; Caruthers, et al., (1982) *Genetic Engineering,* 4:1–17; Jones, chapter 2, Atkinson, et al., chapter 3, and Sproat, et al., chapter 4, in OLIGONUCLEOTIDE SYNTHESIS: A PRACTICAL APPROACH, Gait (ed.), IRL Press, Washington D.C. (1984); Froehler, et al., (1986) *Tetrahedron Lett.,* 27:469–472; Froehler, et al., (1986) *Nucleic Acids Res.,* 14:5399–5407; Sinha, et al. (1983) *Tetrahedron Lett.,* 24:5843–5846; and Sinha, et al., (1984) *Nucl. Acids Res.,* 12:4539–4557, which are incorporated herein by reference.

3. Derivatized Peptides and Peptidomimetics

The design of chemically modified peptides and peptide mimics which are resistant to degradation by proteolytic enzymes or have improved solubility or binding properties is well known.

Modified amino acids or chemical derivatives of GPCRs peptides according to the present invention may contain additional chemical moieties or modified amino acids not normally a part of the protein. Covalent modifications of the peptide are thus included within the scope of the present invention. Such modifications may be introduced into a GPCR polypeptide by reacting targeted amino acid residues of the polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. The following examples of chemical derivatives are provided by way of illustration and not by way of limitation.

The design of peptide mimics which are resistant to degradation by proteolytic enzymes is well known, both for hormone agonist/antagonist and for enzyme inhibitor design. See e.g., Sawyer, in STRUCTURE-BASED DRUG DESIGN, P. Verapandia, Ed., NY 1997; U.S. Pat. No. 5,552,534; and U.S. Pat. No. 5,550,251, all of which are incorporated by reference.

Historically, the major focus of peptidomimetic design has evolved from receptor-targeted drug discovery research and has not been directly impacted by an experimentally-determined three-dimensional structure of the target protein. Nevertheless, a hierarchical approach of peptide→peptidomimetic molecular design and chemical modification has evolved over the past two decades, based on systematic transformation of a peptide ligand and iterative analysis of the structure-activity and structure-conformation relationships of "second generation" analogs. Such work has typically integrated biophysical techniques (x-ray crystallography and/or NMR spectroscopy) and computer-assisted molecular modeling with biological testing to advance peptidomimetic drug design.

The three-dimensional structural properties of peptides are defined in terms of torsion angles (Ψ, ϕ, ω, χ) between the backbone amine nitrogen (N^{α}), backbone carbonyl carbon (C^{1}), backbone methionine carbon (C^{α}), and side chain hydrocarbon functionalization (e.g., C^{β}, C^{λ}, C^{δ}, C^{ϵ} of Lys) derived from the amino acid sequence. A Ramachandran plot (Ψ versus ϕ) may define the preferred combinations of torsion angles for ordered secondary structures (conformations), such as α helix, β turn, γ turn, or β sheet. Molecular flexibility is directly related to covalent and/or noncovalent bonding interactions within a particular peptide. Even modest chemical modifications by N^{α}-methyl, C^{α}-methyl or C^{β}-methyl can have significant consequences on the resultant conformation.

The N^{α}—C^{α}—C' scaffold may be transformed by introduction of olefin substitution (e.g., C^{α}—C^{β}→C═C or dehydroamino acid or insertion (e.g., C^{α}—C'→C^{α}—C═C—C' or vinylogous amino acid. Also the C^{β} carbon may be substituted to advance the design of so-called "chimeric" amino acids Finally, with respect to N-substituted amides it is also noteworthy to mention the intriguing approach of replacing the traditional peptide scaffold by achiral N-substituted glycine building blocks. Overall, such N^{α}—C^{α}—C scaffold or C^{α}—C^{β} side chain modifications expand peptide-based molecular diversity (i.e., so-called "peptoid" libraries) as well as extend our 3-D structural knowledge of traditional ϕ-Ψ-χ space.

In one approach, such as disclosed by Sherman and Spatola, *J. Am. Chem. Soc.* 112: 433 (1990), one or more amide bonds are replaced in an essentially isosteric manner by a variety of chemical functional groups. For example, any amide linkage in any of the GPCR polypeptides can be replaced by a ketomethylene moiety, e.g. (—C(═O)—CH_2—) for (—(C═O)—NH—). A few of the known amide bond replacements include: aminomethylene or $\Psi[CH_2NH]$; ketomethylene or $\Psi[COCH_2]$; ethylene or $\Psi[CH_2CH_2]$; olefin or Ψ[CH═CH]; ether or $\Psi[CH_2O]$; thioether or $\Psi[CH_2S]$; tetrazole or $\Psi[CN_4]$; thiazole or Ψ[thz]; retroamide or Ψ[NHCO]; thioamide or Ψ[CSNH]; and ester or $\Psi[CO_2]$. These amide bond surrogates alter conformational and H-bonding properties that may be requisite for peptide molecular recognition and/or biological activity at receptor targets. Furthermore, such backbone replacements can impart metabolic stability towards peptidase cleavage relative to the parent peptide. The discovery of yet other nonhydrolyzable amide bond isostere has particularly impacted the design of protease inhibitors, and these include: hydroxymethylene or Ψ[CH(OH)]; hydroxyethylene or $\Psi[CH(OH)CH_2]$ and $\Psi[CH_2CH(OH)]$; dihydroxyethylene or Ψ[CH(OH)CH(OH)], hydroxyethylamine or $\Psi[CH(OH)CH_2N]$, dihydroxyethylene and C_2-symmetric hydroxymethylene. Such backbone modifications have been extremely effective, as they may represent transition state mimics or bioisosteres of the hypothetical tetrahedral intermediate (e.g., $\Psi[C(OH)_2NH]$) for this class of proteolytic enzymes. Such derivatives are expected to have the property of increased stability to degradation by enzymes, and therefore possess advantages for the formulation of compounds which may have increased in vivo half lives, as administered by oral, intravenous, intramuscular, intraperitoneal, topical, rectal, intraocular, or other routes.

Both peptide backbone and side chain modifications may provide prototypic leads for the design of secondary structure mimicry, as typically suggested by the fact that substitution of D-amino acids, N^{α}-Me-amino acids, C_{α}-Me amino acids, and/or dehydroamino acids within a peptide lead may induce or stabilize regiospecific β-turn, γ-turn, β-sheet, or α-helix conformations. To date, a variety of secondary structure mimetics have been designed and incorporated in peptides or peptidomimetics. The β-turn has been of particular interest to the area of receptor-targeted peptidomimetic drug discovery. This secondary structural motif exists within a tetrapeptide sequence in which the first and fourth Cα atoms are <7 Å separated, and they are further characterized as to occur in a nonhelical region of the peptide sequence and to possess a ten-membered intramolecular H-bond between the i and i→4 amino acid residues. One of the initial approaches of significance to the design of β-turn mimetics was the monocyclic dipeptide-based template which employs side chain to backbone constraint at the i+1 and i+2 sites. Over the past decade a variety of other monocyclic or bicyclic templates have been developed as β-turn mimetics. Monocyclic β-turn mimetic has been described that illustrate the potential opportunity to design scaffolds that may incorporate each of the side chains (i, i+1, i+2 and i+3 positions), as well as five of the eight NH or C═O functionalities, within the parent tetrapeptide

sequence, tetrapeptide sequence modeled in type I-IV β-turn conformations. Similarly, a benzodiazepine template has shown utility as a β-turn mimetic scaffold which also may be multisubstituted to simulate side chain functionalization, particularly at the i and i+3 positions of the corresponding tetrapeptide sequence modeled in type I–VI β-turn conformations. A recently reported γ-turn mimetic, illustrates an innovative approach to incorporate a retroamide surrogate between the i and i→1 amino acid residues with an ethylene bridge between the N^1 (i.e., nitrogen replacing the carbonyl C') and N atoms of the i and i+2 positions, and this template allows the possibility for all three side chains of the parent tripeptide sequence. Finally, the design of a β-sheet mimetic provides an attractive template to constrain the backbone of a peptide to that simulating an extended conformation. The β-sheet is of particular interest to the area of protease-targeted peptidomimetic drug discovery.

Aromatic amino acids may be replaced with D- or L-napthylalanine, D- or L-phenylglycine, D- or L-2-thieneylalanine, D- or L-1-, 2-, 3- or 4-pyreneylalanine, D- or L-3-thieneylalanine, D- or L-(2-pyridinyl)-alanine, D- or L-(3-pyridinyl)-alanine, D- or L-(2-pyrazinyl)-alanine, D- or L-(4-isopropyl)-phenylglycine, D-(trifluoromethyl)-phenylglycine, D-(trifluoromethyl)-phenylalanine, D-p-fluorophenylalanine, D- or L-p-biphenylphenylalanine, D- or L-p-methoxybiphenylphenylalanine, D- or L-2-indole (alkyl)alanines, and D- or L-alkylainines where alkyl may be substituted or unsubstituted methyl, ethyl, propyl, hexyl, butyl, pentyl, isopropyl, iso-butyl, sec-isotyl, iso-pentyl, non-acidic amino acids, of C_1–C_{20}.

Acidic amino acids can be substituted with non-carboxylate amino acids while maintaining a negative charge, and derivatives or analogs thereof, such as the non-limiting examples of (phosphono)alanine, glycine, leucine, isoleucine, threonine, or serine; or sulfated (e.g., —SO_3H) threonine, serine, tyrosine.

Other substitutions may include unnatural hyroxylated amino acids made by combining "alkyl" (as defined and exemplified herein) with any natural amino acid. Basic amino acids may be substituted with alkyl groups at any position of the naturally occurring amino acids lysine, arginine, ornithine, citrulline, or (guanidino)-acetic acid, or other (guanidino)alkyl-acetic acids, where "alkyl" is define as above. Nitrile derivatives (e.g., containing the CN-moiety in place of COOH) may also be substituted for asparagine or glutamine, and methionine sulfoxide may be substituted for methionine. Methods of preparation of such peptide derivatives are well known to one skilled in the art.

In addition, any amino acid of said peptides can be replaced by the same amino acid but of the opposite chirality. Thus, any amino acid naturally occurring in the L-configuration (which may also be referred to as the R or S, depending upon the structure of the chemical entity) may be replaced with an amino acid of the same chemical structural type, but of the opposite caroled, generally referred to as the D- amino acid but which can additionally be referred to as the R- or the S-, depending upon its composition and chemical configuration. Such derivatives have the property of greatly increased stability co degradation by enzymes, and therefore are advantageous in the formulation of compounds which may have longer in vivo half lives, when administered by oral, intravenous, intramuscular, intraperitoneal, topical, rectal, intraocular, or other routes.

Additional amino acid modifications of amino acids of GPCR polypeptides of to the present invention may include the following: Cysteinyl residues may be reacted with alpha-haloacetates (and corresponding amines), such as 2-chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues may also be derivatized by reaction with compounds such as bromotrifluoroacetone, alpha-bromo-beta-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues may be derivatized by reaction with compounds such as diethylprocarbonate e.g., at pH 5.5–7.0 because this agent is relatively specific for the histidyl side chain, and para-bromophenacyl bromide may also be used; e.g., where the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues may be reacted with compounds such as succinic or other carboxylic acid anhydrides. Derivatization with these agents is expected to have the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing alpha-amino-containing residues include compounds such as imidoesters/ e.g., as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues may be modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin according to known method steps. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

Tyrosyl residues may be modified by reaction with aromatic diazonium compounds or tetranitromethane. N-acetylimidizol and tetranitromethane may be used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl side groups (aspartyl or glutamyl) may be selectively modified by reaction with carbodiimides (R'—N—C—N—R') such as 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore aspartyl and glutamyl residues may be converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues may be frequently deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues may be deamidated under mildly acidic conditions. Either form of these residues falls within the scope of the present invention.

Derivatization with bifunctional agents is useful for cross-linking the peptide to certain chemical moieties. Commonly used cross-linking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl) dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Pat. Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 (which are herein incorporated entirely by reference), may be employed for protein immobilization.

Other modifications of GPCR polypeptides of the present invention may include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains (T. E. Creighton, Proteins: Structure and Molecule Properties, W. H. Freeman & Co., San Francisco, pp. 79–86 (1983)), acetylation of the N-terminal amine, methylation of main chain amide residues (or substitution with N-methyl amino acids) and, in some instances, amidation of the C-terminal carboxyl groups, according to known method steps.

Such derivatized moieties may improve the solubility, absorption, permeability across the blood brain barrier biological half life, and the like. Such moieties or modifications of GPCR polypeptides may alternatively eliminate or attenuate any possible undesirable side effect of the protein and the like. Moleties capable of mediating such effects are disclosed, for example, in Remington's Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, Pa. (1980).

Such chemical derivatives of GPCR polypeptides also may provide attachment to solid supports, including but not limited to, agarose, cellulose, hollow fibers, or other polymeric carbohydrates such as agarose, cellulose, such as for purification, generation of antibodies or cloning; or to provide altered physical properties, such as resistance to enzymatic degradation or increased binding affinity or modulation for GPCRs, which is desired for therapeutic compositions comprising GPCR polypeptides, antibodies thereto or fragments thereof. Such peptide derivatives are well-known in the art, as well as method steps for making such derivatives using carbodiimides active esters of N-hydroxy succinimmide, or mixed anhydrides, as non-limiting examples.

Variation upon the sequences of GPCR polypeptides of the present invention may also include: the addition of one or more (e.g., two, three, four, or five) lysine, arginine or other basic residues or one, or more (e.g., two, three, four, or five) glutamate or aspartate or other acidic residues at one end of the peptide, where "acidic" and "basic" are as defined herein. Negative charges can also be introduced by the addition of carboxyl. phosphate, borate, sulfonate or sulfate groups. Such modifications may increase the alpha-helical content of the peptide by the "helix dipole effect". They also can provide enhanced aqueous solubility of the peptide, and allow the correct insertion of peptides into a membrane structure.

In another approach, a variety of uncoded or modified amino acids such as D-amino acids and N-methyl amino acids have been used to modify mammalian peptides. Alternatively, a presumed bioactive conformation has been stabilized by a covalent modification, such as cyclization or by incorporation of gamma-lactam or other types of bridges. See, e.g., Veber and Hirschmann, et al., *Proc. Natl. Acad. Sci. USA,* 1978 75 2636 and Thorsett, et al., Biochem Biophys. Res. Comm., 1983 111 166. The primary purpose of such manipulations has not been to avoid metabolism or to enhance oral bioavailability but rather to constrain a bioactive conformation to enhance potency or to induce greater specificity for a receptor subtype.

The above examples of peptide scaffold- or nonpeptide template-based peptidomimetic agonists or antagonists illustrate various strategies to elaborate bioactive conformation and/or pharmacophore models of peptide ligands at their receptors. In many cases, receptor subtype selectivity has also been achieved by systematic structural modifications of prototypic leads of peptidomimetics. Thus, although the 3D structures of GPCRs remains elusive (except for models constructed from homology-based low-resolution 3D structures of bacteriorhodopsin or rhodopsin, see below) the development of pharmacophore models using the hierarchial approach in peptide→peptidomimetic structure-based drug design is promising.

4. Purification of GPCR Transmembrane Peptides

The polypeptides of this invention may be purified to substantial purity by standard techniques, including selective precipitation with such substances as ammonium sulfate, column chromatography, immunopurification methods, and others. See, for instance, R. Scopes, *Protein Purification: Principles and Practice*, Springer-Verlag: New York (1982), incorporated by reference. For example, the GPCR transmembrane peptides proteins and polypeptides produced by recombinant DNA technology are purified by a combination of cell lysis (e.g., sonication) and affinity chromatography or immunoprecipitation with a specific antibody to GPCR transmembrane peptides or a peptide fragment thereof. For fusion products, subsequent digestion of the fusion protein with an appropriate proteolytic enzyme releases the desired polypeptide. The proteins may then be further purified by standard protein chemistry techniques. A purified protein preferably exhibits a single band on an electrophoretic gel. Those of skill are reminded that the methods should take into account the hydrophobic nature of the peptides.

5. Detection of GPCR Transmembrane Peptide Gene Products

GPCR transmembrane peptides or a peptide fragment thereof to may be detected or quantified by a variety of methods. Preferred methods involve the use of specific antibodies.

a. Detection of GPCR Transmembrane Peptides by Immunoassay

i. Antibody Production

Methods of producing polyclonal and monoclonal antibodies are known to those of skill in the art. See, e.g., Coligan (1991), Current Protocols in Immunology, Wiley/Greene, NY; and Harlow and Lane (1989), Antibodies: A Laboratory Manual, Cold Spring Harbor Press, NY; Stites et al. (eds.) Basic and Clinical Immunology (4th ed.) Lange Medical Publications, Los Altos, Calif., and references cited therein; Goding (1986), Monoclonal Antibodies: Principles and Practice (2d ed.) Academic Press, New York, N.Y.; and Kohler and Milstein (1975), *Nature,* 256:495–497. Such techniques include antibody preparation by selection of antibodies from libraries of recombinant antibodies in phage or similar vectors. See, Huse et al. (1989), *Science,* 246: 1275–1281; and Ward et al. (1989) *Nature,* 341:544–546. For example, in order to produce antisera for use in an immunoassay, a polypeptide is isolated as described herein. For example, recombinant protein is produced in a transformed cell line. An inbred strain of mice or rabbits is immunized with the peptide using a standard adjuvant, such as Freund's adjuvant, and a standard immunization protocol. Alternatively, a synthetic peptide derived from the sequences disclosed herein and conjugated to a carrier protein can be used an immunogen.

A number of immunogens may be used to produce antibodies specifically reactive with GPCR transmembrane peptides or a peptide fragment thereof. Recombinant protein is the preferred immunogen for the production of monoclonal or polyclonal antibodies. Naturally occurring protein may also be used either in pure or impure form. Synthetic peptides made using the GPCR transmembrane peptides or a peptide fragment thereof sequences described herein may also used as an immunogen for the production of antibodies

to the protein. Recombinant protein can be expressed in eukaryotic or prokaryotic cells as described above, and purified as generally described above. The product is then injected into an animal capable of producing antibodies. Either monoclonal or polyclonal antibodies may be generated, for subsequent use in immunoassays to measure the protein.

Methods of production of polyclonal antibodies are known to those of skill in the art. In brief, an immunogen, preferably a purified protein such as GPCR transmembrane peptides or a peptide fragment thereof is mixed with an adjuvant and injected into an animal of choice (e.g., a mouse, rat, rabbit, pig, goat, cow, horse, chicken, etc.) at intervals of 1–4 weeks. The immunogen may be conjugated to a carrier protein can be used an immunogen. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the GPCR transmembrane peptides or a peptide fragment thereof. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the protein can be done if desired. (See Harlow and Lane, supra).

Polyclonal sera are collected and titered against the immunogen protein in an immunoassay, for example, a solid phase immunoassay with the immunogen immobilized on a solid support. Polyclonal antisera with a titer of 10^4 or greater are selected and tested for their cross reactivity against non-GPCR transmembrane peptides or even GPCR transmembrane peptides from other cell types or species or a peptide fragment thereof, using a competitive binding immunoassay (see, e.g., Harlow and Lane, supra, at pages 570–573). Specific monoclonal and polyclonal antibodies and antisera will usually bind with a K_D of at least about 0.1 mM, more usually at least about 1 μM, preferably at least about 0.1 μM or better, and most preferably, 0.01 μM or better.

Monoclonal antibodies may be obtained by various techniques familiar to those skilled in the art. Briefly, spleen cells from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell (See, Kohler and Milstein, *Eur. J. Immunol.* 6:511–519 (1976), incorporated herein by reference). Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods well known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells may be enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host. Alternatively, one may isolate DNA sequences which encode a monoclonal antibody or a binding fragment thereof by screening a DNA library from human B cells according to the general protocol outlined by Huse, et al. (1989) *Science* 246:1275–1281.

ii. Immunoassays

A particular protein can be measured by a variety of immunoassay methods. For a review of immunological and immunoassay procedures in general, see Basic and Clinical Immunology, 7th Edition (D. Stites and A. Terr, eds.) 1991. Moreover, the immunoassays of the present invention can be performed in any of several configurations, which are reviewed extensively in Enzyme Immunoassay, E. T. Maggio, ed., CRC Press, Boca Raton, Fla. (1980); "Practice and Theory of Enzyme Immunoassays," P. Tijssen, in Laboratory Techniques in Biochemistry and Molecular Biology, Elsevier Science Publishers B.V. Amsterdam (1985); and, Harlow and Lane, Antibodies, A Laboratory Manual, supra, each of which is incorporated herein by reference.

Immunoassays to GPCR transmembrane peptides, peptidomimetics or subfragments thereof may use a polyclonal antiserum raised against a peptide or peptidomimetic of the invention. This antiserum is selected to have low cross-reactivity against other (other non-GPCR transmembrane peptides or other GPCR transmembrane peptides) peptides and any such cross-reactivity is removed by immunoabsorption prior to use in the immunoassay.

Immunoassays in the competitive binding format can be used for the crossreactivity determinations. For example, a reference peptide antigen of the invention can be immobilized to a solid support. The ability of other molecules (other GPCR transmembrane peptides, or non-GPCR transmembrane peptides, or unknowns) to compete with the binding of antisera which recognize the immobilized reference peptide antigen is measured. The ability of such molecules to compete with the binding of an antiserum or antibody to the immobilized reference peptide is compared to a standard molecule, such as the reference peptide antigen itself. The percent crossreactivity is calculated, using standard calculations. Antisera with less than 10% crossreactivity to cross-reacting molecules are selected and pooled. Any cross-reacting antibodies are optionally removed from the pooled antisera by immunoabsorption with cross-reacting molecules.

The immunoabsorbed and pooled antisera are then used in a competitive binding immunoassay to compare the binding of a second protein to that of the reference peptide antigen. In order to make this comparison, the two molecules are each assayed at a wide range of concentrations and the amount of each molecule required to inhibit 50% of the binding of the antisera to the immobilized reference peptide antigen is determined. If the amount of the second protein required is less than 10 times the amount of the reference peptide used to make the antibody, then the second protein is said to specifically bind to an antibody generated to the reference peptide antigen.

The presence of a desired polypeptide (including peptide, translation product, or enzymatic digestion product) in a sample may be detected and quantified using Western blot analysis. The technique generally comprises separating sample products by gel electrophoresis on the basis of molecular weight, transferring the separated proteins to a suitable solid support, (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter), and incubating the sample with labeling antibodies that specifically bind to the analyte protein. The labeling antibodies specifically bind to analyte on the solid support. These antibodies are directly labeled, or alternatively are subsequently detected using labeling agents such as antibodies (e.g., labeled sheep anti-mouse antibodies where the antibody to an analyte is a murine antibody) that specifically bind to the labeling antibody.

6. Detection of GPCR Transmembrane Peptide Sequences, Peptides and Peptidomimetics that Optimally Inhibit GPCR Biological Properties and Functions

Peptides or peptide variants of the invention that modulate biological activity of GPCRs are generally identified as follows. Peptide sequences are selected from the transmembrane domains of the GPCR to be targeted. The transmembrane domains are readily ascertained by the application of computer models to known sequences. Computer modeling and comparison with known transmembrane peptide sequences are also used to define the orientation of the peptide sequence in the membrane, thus allowing the determination of the end of the peptide sequence that is towards

the extracelluar aspect of the plama membrane. The selection of a preferred transmembrane domain to be targeted is largely empirical. We have found that peptides derived from transmembrane domain 2 are particularly effective inhibitors of GPCR function. Alternatively, peptide sequences selected from transmembrane domain 4 have also been effective antagonists.

Upon selection of a peptide sequence, a reference transmembrane sequence is synthesized and systematically modified to identify variants (or analogs) that have improved properties. The modifications introduce a negative charge at the extracellular end of the peptide sequence. Negative charges may be added in the form of acidic amino acid residues such as Asp or Glu. The number of acidic residues that is added is typically from 1 to 3 depending upon the hydrophobicity of the peptide sequence and the subsequent necessity to increase the solubility of the peptide. Further, preferable peptides have a neutral charge at the end of the peptide that is oriented towards the intracellular aspect of the plasma membrane. Thus, the overall hydrophobic nature of such a transmembrane peptide will result in insertion into a membrane and the negative charge at the extracellular end will result in the peptide having the same orientation as the transmembrane GPCR domain from which it is derived. Insertion into the membrane may be tested by fluorescent microscopy of labeled peptide analogs using methodology known to those of skill in the art as illustrated in Example 3 herein.

The ability of the peptide or peptide variants to modulate activity of the targeted GPCR is generally determined by testing the ability of the peptide to inhibit activation that is induced by a natural ligand of the targeted GPCR. Activation of most GPCRs results in an increase in cAMP or the release of intracellular calcium.

Thus, if activation of the target GPCR increases cAMP, the inhibitory activity of the peptide is determined by measuring cAMP levels using methods known to those in the art (see e.g., C. Nordstedt and B. B. Fredholm *Anal. Chem.* 189: 231–234 (1990). Similarly, if activation of the target GPCR releases intracellular calcium, the inhibitory activity of the peptide is determined by measuring the intracellular calcium levels as illustrated in the examples below.

Peptides may be tested for other properties including the following:

- enhanced ability to modulate GPCR activity;
- increased resistance to proteolysis;
- improved solubility;
- longer or shorter half-life, particularly in culture medium or a biological fluid such as plasma or whole blood;
- improved ability to insert into a membrane compartment, especially in a particular orientation, by means known in the art.

Variant peptides may also be synthesized having any one or more of the following modifications:

- conservative or non-conservative substitution of any of the amino acid residues;
- deletion or addition of residues at any position;
- chemical modification at any residue;
- peptidomimetic analogs of the reference peptide.

Variant peptides can be rationally designed and/or screened for using high throughput screening methodologies applied to combinatorial libraries. Methods of generating combinatorial libraries and screening such libraries using high-throughput methods are well known to those of skill in the art (see, e.g., Baum, *C&EN* (Feb. 7, 1994): 20–26 and references cited therein).

These variant peptides are also tested for the any of the above-listed properties. In general, a variant peptide is considered to have improved properties relative to the reference peptide if a given measurable property or parameter associated with the peptide has a value that is at least 10%, preferably at least 30%, more preferably at least 75%, and most preferably at least 95% better than the value for the reference peptide.

The relative ability of the modified peptides (as compared to the reference peptide) to modulate a GPCR biological activity is tested as follows. A cell line that expresses a GPCR and exhibits a GPCR-mediated biological activity is exposed to either the reference or the modified peptide under identical conditions, and the biological property of the GPCR is measured in the absence or presence of either peptide. Examples of cell lines, GPCRs expressed by the cell line, and GPCR-regulated properties measured include the following:

- any cell that stably expresses CXCR4, especially attached cells, including cells that are genetically engineered to express CXCR4, including HeLa cells; CXCR4; stroma cell derived factor I-induced calcium flux;
- any cell that stably expresses CXCR4, especially attached cells, including cells that are genetically engineered to express CXCR4, including CM cells; CXCR4; HIV-1 infection;
- any cell that stably expresses CCKAR, especially attached cells, including cells that are genetically engineered to express CCKAR, such as CHO cells; CCKAR; cholecystokinin-induced calcium release;
- any cell that stably expresses human CCR5, especially attached cells, including cells that are genetically engineered to express CCR5, including HEK cells; CCR5; RANTES induced calcium release.

The inhibitory activity is measured by exposing GPCR-expressing cells to a range of concentrations of a test antagonist, and measuring a biological property or activity associated with that GPCR. The test concentrations can range from 1 nanomolar to 100 micromolar, depending on peptide solubility and affinity. Initial screening is performed using 10-fold dilutions, such as 50, 5, 0.5, 0.05 micromolar. Then, the lowest active concentration is lowered in decrements of 10% to determine the lowest effective concentration. The property measured can be binding to a ligand (for example, binding of cholecystokinin octapeptide to CCKAR), or production of a measurable metabolic response (e.g., altered ion flux or translocation, altered phosphorylation, altered protein synthesis or degradation, altered cellular morphology, altered secretion, altered production of particular components such as soluble inositol phosphates, binding of a virus and subsequent infection, tumor growth, chemotaxis, mitogenic response, cell growth activation, secretion, muscle contraction, vasopressing and vasodepressing activity, synaptic transmission, and release of intracellular calcium, etc.)

The following GPCRs have been reported to play a role in HIV infection:

STRL33	U.S. Provisional Application No. 60/042,880;
CCRR5	U.S. Patent Application No. 60/042,880;
CCR8:	U.S. Provisional Application No. 60/054,094;
CCR2	Proc. Natl. Acad. Sci. USA: 2752–2756 (1994)
	J. Biol. Chem. 270: 29671–29675 (1995)
CCR3:	J. Biol. Chem. 270: 16491–16494 (1995)
CX3CR1:	DNA Cell Biol. 14: 673–680 (1995)

The following is a list of transmembrane peptides that have GPCR antagonist properties:

From the GPCR CXCR4
F-2-2: LLFVITLPFWAVDAVANWYFGNDD (SEQ ID NO:1)

F-2-5: LLFVITLPFWAVDAVANDD (SEQ ID NO:2)

F-4-2: VYVGVWIPALLLTIPDFIFANDD (SEQ ID NO:3)

F-6-1: VILILAFFACWLPYYIGISID (SEQ ID NO:4)

F-7-3: DDEALAFFHCCLNPILYAFL-NH_2 (SEQ ID NO:5)

F-7-4: DDSITEALAFFHCCLNPILYAFL-NH_2 (SEQ ID NO:6)

From the GPCR CCR5
CCR5-TM-2-2: LFFL LTVPFWAHYAAAQWDFGDD (SEQ ID NO:7)

CCR5-TM-4-1: FGVVTSVITWVVAVFASLPGIIFTSSDD (SEQ ID NO:8)

CCR5-TM-6-1: LIFTIMIVYFLFWAPYNIVLLLNTFQED (SEQ ID NO:9)

CCR5-TM-7-1: DDQAMQVTETLGMTHCCINPIIYAFV (SEQ ID NO:10)

From the GPCR CCR2
CCR2-TM-2-1: IYLLNLAISDLLFLITLPLWADD (SEQ ID NO:11)

CCR2-TM-2-2: LLFLITLPLWAH SAANEWVFGNDD (SEQ ID NO:12)

CCR2-TM-4-1: FGVVTSVITWLVAVF ASVPGIIFTDD (SEQ ID NO:13)

CCR2-TM-6-1: VIFTIMIVYFLFWTPYN IVILLNTFQED (SEQ ID NO:14)

CCR2-TM-7-1: DDATQVT ETLGMTHCCINPIIYAFV (SEQ ID NO:15)

From the GPCR CCR3
CCR3-TM-2-1: LLFLVTLPFW IHYVRGHNWVFGDDD (SEQ ID NO:16)

CCR3-TM-4-1: FGVITSIVTWGLAVLAALPEFI FYETED (SEQ ID NO:17)

CCR3-TM-6-1: IFVTMAVFFI FWTPYNVAILLSSYQSDD (SEQ ID NO:18)

CCR3-TM-7-1: DDLVMLVTEVIAYSHCCMNPVIYAFV (SEQ ID NO:19)

From the GPCR CCKAR
CCKAR-TM-1-6: DDEWQSALQILLYSIIFLLSV-LGNTLVITV (SEQ ID NO:20)

CCKAR-TN-2-1: FLLSLAVSDLMLCLFCMPFNLP (SEQ ID NO:21)

CCKAR-TM-2-2: FLLSLAVSDLMLCLFCM PFNLIDD (SEQ ID NO:22)

CCKAR-TM-6-4: IVVLFFLCWMPIFSANAWRAYDTVDD (SEQ ID NO:23)

7. Treatment Embodiments

The compositions containing the present GPCR transmembrane peptides, or a cocktail thereof (i.e., with other molecules, including other peptides of the invention), can be administered for therapeutic treatments. The molecules of the present invention are used to protect a patient from pathologies associated with GPCR, by modulating the biological activities associated with the GPCR. "Protection" from infection or disease as used herein is intended to encompass "prevention" or "treatment." "Treatment" involves administration of the protective composition to a patient exhibiting symptoms of a GPCR-associated pathology (for example, HIV-1 infection), so as to reduce or suppress the symptoms of the pathology. Other examples of GPCR-associated conditions that may be treated with the peptides of the invention include:

cancer. For example, vasoactive intestinal peptide (VIP) receptor is known to be overexpressed in breast cancer and lung cancer, and VIP antagonists are known to inhibit cancer growth. Thus, a peptide of the invention is probably effective in inhibiting such cancers;

antagonists of chemokine receptors as anti-inflamatory and anti asthma drugs;

tissue rejection;

neuropeptide Y receptor antagonists as anti-obesity drugs;

dopamine receptor D4 antagonists as drugs for treatment of depression, attention deficit hyperactivity disorder and schizophrenia;

antagonists of Corticotropin-Releasing Factor Receptor for the treatment of depression and anxiety related disoders;

angiotensin receptor antagonists as a mean of blood pressure control;

antagonists of gastrin-releasing peptide receptor, somatostatin and gastrin receptors as anti-neoplastic agents that slow down growth of endocrine tumors;

antagonists of opiod receptors as pain killers.

a. Pharmaceutical Compositions

The compositions for administration may be in the form of a solution, suspension, tablets, pill, capsule, powder, gel, cream, lotion, ointment, aerosol or the like. In a preferred embodiment, the compositions for administration comprise a solution of the GPCR transmembrane peptides dissolved in

a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., buffeted saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known sterilization techniques. In certain embodiments, the GPCR transmembrane peptides are provided in powder form.

The GPCR transmembrane peptides and analogs may be combined with conventional excipient, such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium, carbonate, and the like. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of the GPCR in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs.

b. Administration and Dosage

The pharmaceutical composition or medium that comprises a GPCR transmembrane peptide is administered orally, parenterally, enterically, gastrically, topically, subcutaneously, rectally, locally or systemically. For example, the compounds can be injected into the bloodstream using a cannula or catheter; the vein or artery is selected to maximize delivery of cells to the affected tissue(s). Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in such publications as *Remington's Pharmaceutical Science,* 15th ed., Mack Publishing Company, Easton, Pa. (1980). It is recognized that the GPCR transmembrane peptides polypeptides and related compounds described above, when administered orally, must be protected from digestion. This is typically accomplished either by complexing the protein with a composition to render it resistant to acidic and enzymatic hydrolysis or by packaging the protein in an appropriately resistant carrier such as a liposome. Means of protecting proteins from digestion are well known in the art.

In therapeutic applications, compositions are administered to a patient suffering from a disease or condition that in an amount sufficient to cure or at least partially arrest symptoms of the disease or conditions and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the condition to be treated and the general state of the patient's health.

Generally, the dosage to be administered is the amount necessary to modulate a GPCR biological activity. It is understood that the dosage of a GPCR polypeptide of the present invention will be dependent upon the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. The ranges of effective doses provided herein are not intended to limit the inventors and represent preferred dose ranges. The most preferred dosage will be tailored to the individual subject, as is understood and determinable by one of skill in the art, without undue experimentation. It is contemplated that the compounds will be administered under the guidance of a physician, who will determine the exact dosages, monitor the progress of the treatment, and determine whether a given administration is successful and sufficient, or whether subsequent administrations are needed.

The concentration of compounds to be administered at a given time and to a given patient will vary from 0.1 μg–100 mg and preferably 0.1–10 mg per day per patient. The dosage and mode of administration may be chosen to achieve and optionally maintain a local concentration in fluids that contact the target cells of about 0.001–50 μg/ml, preferably 0.1–10 μg/ml. Dosages from 0.1 up to about 100 mg per patient per day may be used, particularly when the drug is administered to a secluded site and not into the blood stream, such as into a body cavity or into a lumen of an organ. Substantially higher dosages are possible in topical administration.

Single or multiple administrations of the compositions may be necessary depending on the dosage and frequency as required and tolerated by the patient. In any event, the composition should provide a sufficient quantity of the peptides of this invention to effectively treat the patient.

c. Gene Therapy

The present invention provides packageable GPCR transmembrane peptide-encoding nucleic acids for the transformation of cells in vitro and in vivo. These packageable nucleic acids can be inserted into any of a number of well known vectors for the transfection and transformation of target cells and organisms.

The nucleic acids are transfected into cells, ex vivo or in vivo, through the interaction of the vector and the target cell. The GPCR transmembrane peptide-encoding nucleic acid, under the control of a promoter, then expresses the GPCR transmembrane peptide, thereby modulating the biological activity of a target GPCR.

Such gene therapy procedures have been used to correct acquired and inherited genetic defects, cancer, and viral infection in a number of contexts. The ability to express artificial genes in humans facilitates the prevention and/or cure of many important human diseases, including many diseases which are not amenable to treatment by other therapies. As an example, in vivo expression of cholesterol-regulating genes, genes which selectively block the replication of HIV, and tumor-suppressing genes in human patients dramatically improves the treatment of heart disease, AIDS, and cancer, respectively. For a review of gene therapy procedures, see Anderson, *Science* (1992)256:808–813; Nabel and Feigner (1993) *TIBTECH* 11: 211–217; Mitani and Caskey (1993) TIBTECH 11: 162–166; Mulligan (1993) *Science* 926–932; Dillon (1993) *TIBTECH* 11: 167–175; Miller (1992) *Nature* 357: 455–460; Van Brunt (1988) *Biotechnology* 6(10): 1149–1154; Vigne (1995) *Restorative Neurology and Neuroscience* 8: 35–36; Kremer and Perricaudet (1995) *British Medical Bulletin* 51(1) 31–44; Haddada et al., (1995) in Current Topics in Microbiology and Immunology Doerfler and Böhm (eds) Springer-Verlag, Heidelberg Germany; and Yu et al., Gene Therapy (1994) 1:13–26.

Delivery of the gene or genetic material into the cell is the first critical step in gene therapy treatment of disease. A large number of delivery methods are well known to those of skill in the art. Such methods include, for example liposome-based gene delivery (Debs and Zhu (1993) WO 93/24640; Mannino and Gould-Fogerite (1988) *BioTechniques* 6(7): 682–691; Rose U.S. Pat. No. 5,279,833; Brigham (1991) WO 91/06309; and Felgner et al. (1987) *Proc. Natl. Acad. Sci. USA* 84: 7413–7414), and replication-defective retroviral vectors harboring a therapeutic polynucleotide sequence as part of the retroviral genome (see, e.g., Miller et al. (1990)

Mol. Cell. Biol. 10:4239 (1990); Kolberg (1992) *J. NIH Res.* 4:43, and Cornetta et al. *Hum. Gene Ther.* 2:215 (1991)). Widely used retroviral vectors include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), Simian Immuno deficiency virus (SIV), human immuno deficiency virus (HIV), and combinations thereof. See, e.g., Buchscher et al.-(1992) *J. Virol.* 66(5) 2731–2739; Johann et al. (1992) *J. Virol.* 66 (5):1635–1640 (1992); Sommerfelt et al., (1990) *Virol.* 176:58–59; Wilson et al. (1989) *J. Virol.* 63:2374–2378; Miller et al., *J. Virol.* 65:2220–2224 (1991); Wong-Staal et al., PCT/US94/05700, and Rosenburg and Fauci (1993) in *Fundamental Immunology, Third Edition* Paul (ed) Raven Press, Ltd., New York and the references therein, and Y et al., GENE THERAPY (1994), supra).

AAV-based vectors are also used to transduce cells with target nucleic acids, e.g., in the in vitro production of nucleic acids and peptides, and in in vivo and ex vivo gene therapy procedures. See, West et al. (1987) *Virology* 160:38–47; Carter et al. (1989) U.S. Pat. No. 4,797,368; Carter et al. WO 93/24641 (1993); Kotin (1994) *Human Gene Therapy* 5:793–801; Muzyczka (1994) *J. Clin. Invest.* 94:1351 and Samulski (supra) for an overview of AAV vectors. Construction of recombinant AAV vectors are described in a number of publications, including Lebkowski, U.S. Pat. No. 5,173,414; Tratschin et al. (1985) *Mol. Cell. Biol.* 5(11):3251–3260; Tratschin, et al. (1984) *Mol. Cell. Biol.*, 4:2072–2081; Hermonat and Muzyczka (1984) *Proc. Natl. Acad. Sci. USA,* 81:6466–6470; McLaughlin et al. (1988) and Samulski et al. (1989) *J. Virol.*, 63:03822–3828. Cell lines that can be transformed by rAAV include those described in Lebkowski et al. (1988) *Mol. Cell. Biol.*, 8:3988–3996.

i. In Vitro Gene Transfer

It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression of DNA encoding GPCR transmembrane peptides or a peptide fragment thereof. No attempt to describe in detail the various methods known for the expression of proteins in prokaryotes or eukaryotes is made here.

There are several well-known methods of introducing nucleic acids into bacterial and animal cells, any of which may be used in the present invention. These include: calcium phosphate precipitation, fusion of the recipient cells with bacterial protoplasts containing the DNA, treatment of the recipient cells with liposomes containing the DNA, DEAE dextran, receptor-mediated endocytosis, electroporation, micro-injection of the DNA directly into the cells, infection with viral vectors, etc.

For in vitro applications, the delivery of nucleic acids can be to any cell grown in culture, whether of bacterial, plant or animal origin, vertebrate or invertebrate, and of any tissue or type. Contact between the cells and the genetically engineered nucleic acid constructs, when carried out in vitro, takes place in a biologically compatible medium. The concentration of nucleic acid varies widely depending on the particular application, but is generally between about 1 μmol and about 10 mmol. Treatment of the cells with the nucleic acid is generally carried out at physiological temperatures (about 37° C.) for periods of time of from about 1 to 48 hours, preferably of from about 2 to 4 hours.

In one group of embodiments, a nucleic acid is added to 60–80% confluent plated cells having a cell density of from about 10^3 to about 10^5 cells/mL, more preferably about 2×10^4 cells/mL. The concentration of the suspension added to the cells is preferably of from about 0.01 to 0.2 μg/mL, more preferably about 0.1 μg/mL.

ii. In vivo Gene Transfer

Alternatively, the GPCR transmembrane peptide encoding nucleic acids can also be introduced into target cells in vivo, using recombinant methods which are known to those of skill in the art. The insertion of genes into cells for the purpose of medicinal therapy is a rapidly growing field in medicine which has enormous clinical potential. Research in gene therapy has been on-going for several years, and has entered human clinical trials. Zhu, et al., *Science,* 261: 209–211 (1993), incorporated herein by reference, describes the intravenous delivery of cytomegalovirus (CMV)-ch loramphenicol acetyltransferase (CAT) expression plasmid using DOTMA-DOPE complexes. Hyde, et al., *Nature,* 362:250–256 (1993), incorporated herein by reference, describes the delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene to epithelia of the airway and to alveoli in the lung of mice, using liposomes. Brigham, et al., *Am. J. Med. Sci.,* 298:278–281 (1989), incorporated herein by reference, describes the in vivo transfection of lungs of mice with a functioning prokaryotic gene encoding the intracellular enzyme chloramphenicol acetyltransferase (CAT).

Formulations suitable for administration include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations of packaged nucleic acid can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials. Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

For in vivo administration, pharmaceutical compositions that comprise GPCR transmembrane peptide-encoding nucleic acids are preferably administered parenterally, i.e., intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. More preferably, the pharmaceutical compositions are administered intravenously or intraperitoneally by a bolus injection. For example, see Stadler, et al., U.S. Pat. No. 5,286,634, which is incorporated herein by reference. Intracellular nucleic acid delivery has also been discussed in Straubringer, et al., *Methods in Enzymology*, Academic Press, New York. 101:512–527 (1983); Mannino, et al., *Biotechniques,* 6:682–690 (1988); Nicolau, et al., *Crit. Rev. Ther. Drug Carrier Syst.,* 6:239–271 (1989), and Behr, *Acc. Chem. Res.,* 26:274–278 (1993). Still other methods of administering therapeutics are described in, for example, Rahman et al., U.S. Pat. No. 3,993,754; Sears, U.S. Pat. No. 4,145,410; Papahadjopoulos et al., U.S. Pat. No. 4,235,871; Schneider, U.S. Pat. No. 4,224,179; Lenk et al., U.S. Pat. No. 4,522,803; and Fountain et al., U.S. Pat. No. 4,588,578.

In preferred embodiments, the pharmaceutical preparations may be contacted with the target tissue by direct application of the preparation to the tissue. The application may be made by topical, "open" or "closed" procedures. By "topical", it is meant the direct application of the pharmaceutical preparation to a tissue exposed to the environment, such as the skin, oropharynx, external auditory canal, and the like. "Open" procedures are those procedures which include incising the skin of a patient and directly visualizing the underlying tissue to which the pharmaceutical preparations are applied. This is generally accomplished by a surgical procedure, such as a thoracotomy to access the lungs, abdominal laparotomy to access abdominal viscera,

or other direct surgical approach to the target tissue. "Closed" procedures are invasive procedures in which the internal target tissues are not directly visualized, but accessed via inserting instruments through small wounds in the skin. For example, the preparations may be administered to the peritoneum by needle lavage. Likewise, the preparations may be administered through endoscopic devices.

The nucleic acid can also be administered in an aerosol inhaled into the lungs (see, Brigham, et al., *Am. J. Sci.*, 298(4):278–281 (1989)) or by direct injection at the site of disease (Culver, *Human Gene Therapy*, MaryAnn Liebert, Inc., Publishers, New York. pp. 70–71 (1994)).

Effective doses of the compositions of the present invention will vary depending upon many different factors, including means of administration, target site, physiological state of the patient, and other medicants administered. Thus, treatment dosages will need to be titrated to optimize safety and efficacy. In determining the effective amount of the vector to be administered, the physician evaluates the particular nucleic acid used, the disease state being diagnosed; the age, weight, and condition of the patient, circulating plasma levels, vector toxicities, progression of the disease, and the production of anti-vector antibodies. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular vector. Doses ranging from about 10 ng to 1 g, 100 ng to 100 mg, 1 μg to 10 mg, or 30–300 μg DNA per patient are typical. Doses generally range between about 0.01 and about 50 mg per kilogram of body weight; preferably between about 0.1 and about 5 mg/kg of body weight or about 10^8–10^{10} or 10^{12} particles per injection. In general, the dose equivalent of a naked nucleic acid from a vector is from about 1 μg to 100 μg for a typical 70 kilogram patient, and doses of vectors which include a retroviral particle are calculated to yield an equivalent amount of inhibitor nucleic acid.

Prior to infusion, blood samples are obtained and saved for analysis. Between 10^8 and 1×10^{12} vectors are infused intravenously over 60–200 minutes. Vital signs and oxygen saturation by pulse oximetry are closely monitored. Blood samples are obtained 5 minutes and 1 hour following infusion and saved for subsequent analysis. At the physician's discretion, reinfusion is repeated every 2 to 3 months for a total of 4 to 6 treatments in a one year period. After the first treatment, infusions can be performed on a outpatient basis at the discretion of the clinician. If the reinfusion is given as an outpatient, the participant is monitored for at least 4, and preferably 8 hours following the therapy.

If a patient undergoing infusion of a vector or transduced cell develops fevers, chills, or muscle aches, he/she receives the appropriate dose of aspirin, ibuprofen or acetaminophen. Patients who experience reactions to the infusion such as fever, muscle aches, and chills are premedicated 30 minutes prior to the future infusions with either aspirin, acetaminophen, or diphenhydramine. Meperidine is used for more severe chills and muscle aches that do not quickly respond to antipyretics and antihistamines. Vector infusion is slowed or discontinued depending upon the severity of the reaction.

In vivo gene transfer may be practiced in a variety of hosts. Preferred hosts include mammalian species, such as humans, non-human primates, dogs, cats, cattle, horses, sheep, and the like.

EXAMPLES

The following examples are simply embodiments of the invention and are not intended to limit the invention. A person of ordinary skill in the art can modify and/or adapt the invention for various applications without undue experimentation, without departing from the generic concept of the present invention. Therefore, such adaptations and modifications are within the scope and range of the present invention.

Example 1

Example 1 illustrates that peptides derived from transmembrane regions of CXCR4 inhibit CXCR4-mediated calcium fluxes.

Peptides having the selected sequences were synthesized by a flow-through solid phase peptide synthesis on 432A Applied Biosystems Peptide Synthesizer utilizing Fmoc amino acid derivatives. To overcome the aggregation that frequently occurs during the synthesis of hydrophobic peptides and leads to the blockage of the growing peptide chain, FmocHmb derivatives of Ala, Val and Leu were introduced into the difficult sequences. Charged residues were added to the peptide termini to assure a proper orientation of the peptides during penetration into the cellular membrane, and to improve the solubility of the highly hydrophobic peptides.

The purity of the peptides was assessed by reverse phase HPLC and the structures were confirmed by matrix-assisted laser-desorption time-of-flight (MALDI-TOF) mass spectrometry (Tarasova et al. (1998), *Ad. Exp. Med. Biol.*, Plenum Press, NY, pp. 201–206.)

Peptides used in this example are listed in Tables 1, 2, and 3.

The effect of the peptides on CXCR4-mediated calcium fluxes in HeLa cells that naturally express the CXCR4 receptor and U87 cells stably expressing the CXCR4 receptor was tested as follows. Cells grown on Nunc cover glass chamber slides were incubated with 1 micromolar Fura-2/AM for 20 min in a CO_2-incubator, rinsed with PBS and mounted on the stage of a Zeiss Axiovert inverted microscope. $[Ca^{2+}]_i$ measurements were performed using an Attofluor digital imaging system (Atto Instruments, Rockville, Md.). Fluorescence of Fura was excited at alternating wavelength of 340 and 380 nm. Fluorescence was monitored by an intensified CCD camera using a 505 cut-off filter. Calibrations of $[Ca^{2+}]_i$ signals were performed using Ca^{2+} standards containing 1 micromolar Fura. CXCR4 antagonists were tested on HeLa cells and U87 cells. Stromal cell-derived factor-1α (SDF-1α) was used as a specific CXCR4 agonist.

CCR5 antagonists, which were used in selectivity studies as described below, were tested on HEK (human kidney carcinoma) cells stably expressing the CCR5 receptor and RANTES was used as an agonist. The antagonist activity of the peptides was evaluated by measuring the inhibition of agonist-evoked intracellular Ca^{2+}i release. These measurements were carried out in Fura-2/AM-treated cells, utilizing an Attofluor digital imaging system as described above. The agonist was SDF-1 α.

In the preliminary screen, peptides corresponding to the second and sixth transmembrane domains were found to abolish SDF-1α-induced signaling through CXCR4 receptor (Table 1). Further optimization and structure-activity studies allowed to obtain antagonists derived from all but the third and fifth transmembrane domains (Table 2).

TABLE 1

Activity of synthetic peptides corresponding to predicted transmembrane domains of CXCR4 in inhibition of SDF-1α-induced intracellular calcium release.

Peptide	Concentration, required for complete inhibition of $[Ca^{2+}_I]$ release
F-1-5: DDIFLPTIYSIIFLTGIV-HN_2 (SEQ ID NO:350)	>30 μM
F-2-1: LLFVITLPFWAVDAVANWYFGN (SEQ ID NO:351)	5 μM
F-3-1: KAVHVIYTVNLYSSVLILAFISL-NH_2 (SEQ ID NO:352)	>50 μM
F-4-1: KVYVGVWIPALLLTLPDFIF (SEQ ID NO:353)	>50 μM
F-5-1: HIMVGLILPGIVILSCYCIII-NH_2 (SEQ ID NO:354)	>50 μM
F-6-1: VILILAFFACWLPYYIGISID (SEQ ID NO:4)	10 μM
F-7-1: ALAFFHCCLNPILYAFLGAK-NH_2 (SEQ ID NO:355)	>100 μM

TABLE 2

Biological activity of CXCR4 antagonists derived from different transmembrane domains. Anti signaling activity was determined in inhibition of SDF-1α-induced intracellular calcium release. Anti-HIV-1 activity was assessed in cytoprotection assay utilizing CEM-SS cells infected with $HIV\text{-}1_{RF}$.

Peptide	Concentration, required for inhibition of signal transduction (μM)	EC_{50} in anti-HIV-1 assay (μM)
F-2-2 LLFVITLPFWAVDAVANWYFGNDD (SEQ IDNO:1)	0.2	2.27
F-4-2 VYVGVWIPALLLTIPDFIFANDD (SEQ IDNO:3)	5	0.3
F-6-1 VILILAFFACWLPYYIGISID (SEQ IDNO:4)	10	>50
F-7-3 DDEALAFFHCCLNPILYAFL-NH_2 (SEQ IDNO:5)	25	3.27
F-6-1 + F-7-3	1	No data

To further understand the structural requirements for a successful antagonist, structure-activity studies were conducted on the peptides derived from the second transmembrane domain of CXCR4 (Table 3). The most potent antagonist, a 24 amino acid residue peptide F-2-2, completely blocked signal transduction at 0.2 micromolar. Addition of negatively charged residues to the termini appeared to be important for the activity. Elimination of the added negative charges provided by two C-terminal Asp residues (F-2-1) decreased antagonist potency more than ten-fold. Consistent with those findings, the substitution of negatively charged aspartate residues with positively charged lysines (F-2-3) resulted in 100-fold dicrease in antagonist activity. Deletion of five residues preceding the C-terminal aspartates (F-2-4) reduced the potency 20-fold. Truncation of the transmembrane portion by three N-terminal residues Leu-Leu-Phe rendered the peptide inactive.

TABLE 3

Structure-activity relationships in peptides derived from the second transmembrane domain of CXCR4: ...HLSVADLLFVITLPFWAVDAVANWYFGNFLCK... (SEQ ID NO:356) (predicted intramembrane portion is underlined)

Peptide	Concentration, required for complete inhibition of $[Ca^{2+}_I]$ release
F-2-1: LLFVITLPFWAVDAVANWYFGN (SEQ ID NO:351)	5 μM
F-2-2: LLFVITLPFWAVDAVANWYFGNDD (SEQ ID NO:1)	0.2 μM
F-2-8: LLFVITLPFWAVDAVANWYFGNKK (SEQ ID NO:357)	20 μM
F-2-4: VITLPFWAVDAVANWYFGNKK (SEQ ID NO:358)	>50 μM
F-2-5: LLFVITLPFWAVDAVANDD (SEQ ID NO:2)	10 μM
AcF-2-5: AcLLFVITLPFWAVDAVANDD (SEQ ID NO:359)	10 μM
F-2-6: LSVADLLFVITLPFWAVDAVANDD (SEQ ID NO:360)	20 μM
Rhod - AcF-2-2: AcLLFVITLPFWAVDAVANWYFGNDDK(Rhod)D (SEQ ID NO:361)	8 μM

Similar results were also observed for peptides derived from additional transmembrane regions. For example, in the case of peptides corresponding to the fourth transmembrane domain, positioning of the charged residue at the intracellular end of the peptide (F4-1, Table 1) instead of the extracellular end (F-4-2, Table 2) abolished the antagonist activity. Further, substitution of extracellular aspartates with lysines also abolished the antagonist activity (data not shown).

The specificity of the transmembrane domain interaction was demonstrated by the fact that all peptides derived from CXCR4 showed selectivity for that receptor and had no influence on signaling of the other chemokine receptor involved in HIV-1 entry, CCR5. Similarly, a peptide derived from the second transmembrane domain of CCR5, LFFLLTVPFWAHYAAAQWDFGDD (SEQ ID NO:7), completely abolished agonist induced signaling of the receptor in U87 cells at 500 nM concentrations, but had no effect on signaling of CXCR4.

It was further noted that an equimolar mixture of two peptides, F-6-1 and F-7-3, was an order of magnitude more potent than the most active of the two peptides. This synergistic effect produced by the derivatives of the sixth and seventh transmembrane regions may be a general phe-

nomenon. Thus, pairs of TM analogs in optimized combinations may act as very potent antagonists.

Example 2

Example 2 illustrates that synthetic peptides corresponding to transmembrane domains of CXCR4 inhibit CXCR4-mediated HIV infection.

CCR5 and CXCR4 are believed to be the main co-receptors for HIVI cell entry (Broder et al., (1997), *J. Leukoc. Biol.* 62:2029; Doranz et al. (1997) *Immunol. Res.* 16: 1528; Premack and Schall (1996), *Nat. Med.* 2:11741178.), although other chemokine receptors appear to mediate infection as well (Michael et al. (1997) *Nat. Med.* 3(10):11602).

The ability of synthetic CXCR4-derived peptides of Table 1 to inhibit HIV-1 infection of CEM-SS cells was tested using an LAV strain of the virus that is known to utilize CXCR4 as a co-receptor. Anti-HIV-1 assay. Buckheit et al. (1993) Antiviral Research 21: 247. The CEM-SS cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum. The cells were placed in each well of a 96-well microtiter plate to a density of 5×10^3 cells per well. The cells were infected with HIV-1 virus at a multiplicity of infection (MOI) previously determined to produce maximal level of viral production at 6 days post infection (MOI of 0.01).

Serial half-log dilutions of test compound were added to appropriate wells in triplicate to evaluate their ability to inhibit HIV-1 infection. AZT was used in parallel as a positive control. Following 6 days of incubation at 37° C., the presence and relative abundance of viral p24 protein was determined by ELISA in cell-free supernatants derived from each well of the microtiter plate. The p24 ELISA kit was purchased from the AIDS Vaccine Program, NCI, FCRDC (Frederick, Md.) and the assay was performed according to the manufacturer's instructions.

Most of the peptides in Table 1 showed some antiviral activity (data not shown). However, the peptides corresponding to the second and sixth transmembrane domains were the most potent in inhibition of HIV entry. The F-2-2 compound completely inhibited infection at a 5 micromolar concentration (FIG. **1**).

Peptides corresponding to transmembrane domains of the cholecystokinin type A receptor (CCKAR) were used as negative controls and did not effect CXCR4 function, thereby confirming the specificity of the effect.

The peptides showed no cell toxicity in concentrations up to 100 micromolar (higher concentrations could not be tested because of solubility problems).

The ability of synthetic peptides to inhibit HIV-1 infection was additionally tested by cytoprotection assay using the highly cytopathic HIV-1 strain RF (Rice, et al. (1995) *Adv. Pharmacol.* 33:389,) (Table 2). The most potent peptide, F-4-2, completely inhibited infection at 1 micromolar concentration (FIG. **2**). The peptides used as negative controls, which correspond to transmembrane domains of the cholecystokinin receptor type A, did not effect chemokine receptors functions.

The above results generally demonstrate the ability of externally added molecules to compete for interaction between transmembrane domains of GPCRs and thereby to disrupt receptor function (FIG. **3**). In addition, it is important to note that the peptides of the invention inhibit HIV infection by targeting a cellular molecule and function rather than a viral molecule and function. Viral proteins have a relatively high mutation rate, which often allows viruses to become resistant to a given treatment. Because cellular proteins mutate at a far slower rate, the probability that a virus will be able to develop a resistance is greatly reduced.

Example 3

Example 3 shows that the peptides of the invention partition to the plasma membrane and other membrane compartments.

A fluorescent derivative of CXCR4 TM2, rhodamine-F-2, was synthesized by solid-phase synthesis on 432A Applied Biosystems Peptide Synthesizer utilizing Fmoc amino acid derivatives. Rhodamine B (Fluka) was loaded onto the amino acid column of the instrument. The purity of the peptide was assessed by reverse phase HPLC and the structures were confirmed by matrix-assisted laser desorption mass spectrometry.

A chimeric protein consisting of the CXCR4 and the green fluorescent protein (GFP) was used for studying receptor localization, internalization, and recycling in live cells in real time. This construct was made and stably expressed in HeLa cells as described in Tarasova et al. (1997), *J. Biol. Chem.* 272: 14817–14824. Fusion of the C terminus of the CXCR4 to the N terminus of the GFP did not appear to alter receptor ligand binding affinity, signal transduction, or the pattern of receptor surface expression and distribution.

Transfected CXCR4-GFP-expressing HeLa cells were grown in coated 50 mm cover glass bottom dishes (MatTek, MA) in medium without phenol red. The cells were then exposed to 1 micromolar peptide in DMEM medium for 30 min in a CO_2-incubator. The distribution of fluorescent label was determined by confocal laser scanning microscopy on a Zeiss inverted LSM 410 laser scanning confocal microscope. Fluorescence of GFP was excited using a 488 nm argon/krypton laser; emitted fluorescence was detected with 515–540 nm bandpass filter. For rhodamine red a 568 nm helium/neon laser was used for excitation and fluorescence was detected with a 590–640 nm bandpass filter.

The results demonstrated that the rhodaminated peptide co-localized with the CXCR4-GFP and was present at the cellular membrane within minutes after application and saturated endosomes and the endopolasmic reticulum after 15 minutes of incubation. This confirmed the ability of the peptides to concentrate in the cellular membranes and suggested that the peptides interacted with receptor molecules.

Example 4

Example 4 shows that peptides corresponding to transmembrane domains of the cholecystokinin type A receptor (CCKAR) inhibits agonist-evoked intracellular calcium release with a potency similar to CXCR4 compounds.

To further illustrate the present invention, we have synthesized peptides derived from the transmembrane domains of the rat cholecystokinin receptor type A (CCKAR). Although CCKAR belongs to the same rhodopsin family of GPCRs as CSCR4, its sequence is only 15% identical to that of CXCR4, when aligned using the Dialign 2 program (Morgenstern, et al,. (1996) *Proc. Natl. Acad. Sci. USA* 93:12098) and the degree of identify in transmembrane regions is only ^{27}%.

The activity of peptides from the CCKAR transmembrane domain were tested in transfected CHO cells that stably express rat CCKAR (Tarasova et al. (1997), *J. Biol. Chem.* 272: 14817–14824). Sulfated cholecystokinin octapeptide was the CCKAR agonist. Determination of intracellular calcium release was performed as described in example 1 above. The results are shown in Table 4.

None of CCKAR-derived peptides served as antagonists of chemokine receptors and none could inhibit HIV-1 infection. The activity of the antagonists in inhibiting signaling through the receptor was compared to the ability to prevent agonist binding (Table 4). Inhibition of signaling was assessed in CCK-8 evoked intracellular calcium release in FURA-2/AM treated CHO cells stably transfected with rat

CCKAR (Tarasova, et al. (1997) *J. Biol. Chem.* 272:14817). Inhibition of ligand binding was measured with the use of a fluorescent agonist, rhodamine green CCK-8 (RG-CCK-8) and quantitative confocal laser scanning microscopy (Tarasova, et al. (1997) *J. Biol. Chem.* 272:14817). Peptides derived from the first, second, and sixth transmembrane domains inhibited CCK-induced signaling through the CCKAR receptor. Peptides derived from the first and the second transmembrane domains, CCKAR-1-1 and CCKAR-2-1, had comparable potencies with respect to the inhibition of ligand signaling and binding. A peptide derived from the sixth domain, CCKAR-6-1, was active in inhibition of signaling, but had very low activity in inhibition of RG-CCK-8 binding.

TABLE 4

The activity of CCKAR-derived TM peptides in inhibition of CCK-8 -induced intracellular calcium release and RG-CCK-8 binding.

Peptide	Concentration, required for inhibition of signaling	IC_{50} in inhibiton of RG-CCK-8 binding
CCKAR-TM-1-6: DDEWQSALQILLYSIIFLLSVLGNTLVITV (SEQ ID NO:20)	50 μM	20 μM
CCKAR-TM-2-1: FLLSLAVSDLMLCLFCMIPFNLP (SEQ ID NO:21)	2 μM	0.5 μM
CCKAR-TM-4-2 (#71) VIAATWCLSFTLMTPYPIYSNLVPFTDD (SEQ ID NO:362)	>50 μM	>50 μM
CCKAR-TM-5-3 (#45) DDQTFLLLILFLLPGIVMVVAYGL (SEQ ID NO:363)	>50 μM	>50 μM
CCKAR-TM-6-4 (#77) IVVLFFLCWMPIFSANAWRAYDTVDD SEQ ID NO:23	5 μM	>50 μM

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 363

<210> SEQ ID NO 1
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: F-2-2 GPCR CXCR4

<400> SEQUENCE: 1

Leu Leu Phe Val Ile Thr Leu Pro Phe Trp Ala Val Asp Ala Val Ala
1 5 10 15

Asn Trp Tyr Phe Gly Asn Asp Asp
20

<210> SEQ ID NO 2
<211> LENGTH: 19
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: F-2-5 GPCR CXCR4

<400> SEQUENCE: 2

Leu Leu Phe Val Ile Thr Leu Pro Phe Trp Ala Val Asp Ala Val Ala
1 5 10 15

Asn Asp Asp

-continued

<210> SEQ ID NO 3
<211> LENGTH: 23
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: F-4-2 GPCR CXCR4

<400> SEQUENCE: 3

Val Tyr Val Gly Val Trp Ile Pro Ala Leu Leu Leu Thr Ile Pro Asp
1 5 10 15

Phe Ile Phe Ala Asn Asp Asp
20

<210> SEQ ID NO 4
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: F-6-1 GPCR CXCR4

<400> SEQUENCE: 4

Val Ile Leu Ile Leu Ala Phe Phe Ala Cys Trp Leu Pro Tyr Tyr Ile
1 5 10 15

Gly Ile Ser Ile Asp
20

<210> SEQ ID NO 5
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: F-7-3 GPCR CXCR4
<221> NAME/KEY: MOD_RES
<222> LOCATION: (20)...(20)
<223> OTHER INFORMATION: Xaa = leucinamide

<400> SEQUENCE: 5

Asp Asp Glu Ala Leu Ala Phe Phe His Cys Cys Leu Asn Pro Ile Leu
1 5 10 15

Tyr Ala Phe Xaa
20

<210> SEQ ID NO 6
<211> LENGTH: 23
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: F-7-4 GPCR CXCR4
<221> NAME/KEY: MOD_RES
<222> LOCATION: (23)...(23)
<223> OTHER INFORMATION: Xaa = leucinamide

<400> SEQUENCE: 6

Asp Asp Ser Ile Thr Glu Ala Leu Ala Phe Phe His Cys Cys Leu Asn
1 5 10 15

Pro Ile Leu Tyr Ala Phe Xaa
20

<210> SEQ ID NO 7
<211> LENGTH: 23
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCR5-TM-2-2 GPCR CCR5

-continued

<400> SEQUENCE: 7

Leu Phe Phe Leu Leu Thr Val Pro Phe Trp Ala His Tyr Ala Ala Ala
1 5 10 15

Gln Trp Asp Phe Gly Asp Asp
20

<210> SEQ ID NO 8
<211> LENGTH: 28
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCR5-TM-4-1 GPCR CCR5

<400> SEQUENCE: 8

Phe Gly Val Val Thr Ser Val Ile Thr Trp Val Val Ala Val Phe Ala
1 5 10 15

Ser Leu Pro Gly Ile Ile Phe Thr Ser Ser Asp Asp
20 25

<210> SEQ ID NO 9
<211> LENGTH: 28
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCR5-TM-6-1 GPCR CCR5

<400> SEQUENCE: 9

Leu Ile Phe Thr Ile Met Ile Val Tyr Phe Leu Phe Trp Ala Pro Tyr
1 5 10 15

Asn Ile Val Leu Leu Leu Asn Thr Phe Gln Glu Asp
20 25

<210> SEQ ID NO 10
<211> LENGTH: 26
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCR5-TM-7-1 GPCR CCR5

<400> SEQUENCE: 10

Asp Asp Gln Ala Met Gln Val Thr Glu Thr Leu Gly Met Thr His Cys
1 5 10 15

Cys Ile Asn Pro Ile Ile Tyr Ala Phe Val
20 25

<210> SEQ ID NO 11
<211> LENGTH: 23
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCR2-TM-2-1 GPCR CCR2

<400> SEQUENCE: 11

Ile Tyr Leu Leu Asn Leu Ala Ile Ser Asp Leu Leu Phe Leu Ile Thr
1 5 10 15

Leu Pro Leu Trp Ala Asp Asp
20

<210> SEQ ID NO 12
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCR2-TM-2-2 GPCR CCR2

-continued

<400> SEQUENCE: 12

Leu Leu Phe Leu Ile Thr Leu Pro Leu Trp Ala His Ser Ala Ala Asn
1 5 10 15

Glu Trp Val Phe Gly Asn Asp Asp
20

<210> SEQ ID NO 13
<211> LENGTH: 26
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCR2-TM-4-1 GPCR CCR2

<400> SEQUENCE: 13

Phe Gly Val Val Thr Ser Val Ile Thr Trp Leu Val Ala Val Phe Ala
1 5 10 15

Ser Val Pro Gly Ile Ile Phe Thr Asp Asp
20 25

<210> SEQ ID NO 14
<211> LENGTH: 28
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCR2-TM-6-1 GPCR CCR2

<400> SEQUENCE: 14

Val Ile Phe Thr Ile Met Ile Val Tyr Phe Leu Phe Trp Thr Pro Tyr
1 5 10 15

Asn Ile Val Ile Leu Leu Asn Thr Phe Gln Glu Asp
20 25

<210> SEQ ID NO 15
<211> LENGTH: 25
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCR2-TM-7-1 GPCR CCR2

<400> SEQUENCE: 15

Asp Asp Ala Thr Gln Val Thr Glu Thr Leu Gly Met Thr His Cys Cys
1 5 10 15

Ile Asn Pro Ile Ile Tyr Ala Phe Val
20 25

<210> SEQ ID NO 16
<211> LENGTH: 25
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCR3-TM-2-1 GPCR CCR3

<400> SEQUENCE: 16

Leu Leu Phe Leu Val Thr Leu Pro Phe Trp Ile His Tyr Val Arg Gly
1 5 10 15

His Asn Trp Val Phe Gly Asp Asp Asp
20 25

<210> SEQ ID NO 17
<211> LENGTH: 28
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCR3-TM-4-1 GPCR CCR3

-continued

<400> SEQUENCE: 17

Phe Gly Val Ile Thr Ser Ile Val Thr Trp Gly Leu Ala Val Leu Ala
1 5 10 15

Ala Leu Pro Glu Phe Ile Phe Tyr Glu Thr Glu Asp
20 25

<210> SEQ ID NO 18
<211> LENGTH: 28
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCR3-TM-6-1 GPCR CCR3

<400> SEQUENCE: 18

Ile Phe Val Ile Met Ala Val Phe Phe Ile Phe Trp Thr Pro Tyr Asn
1 5 10 15

Val Ala Ile Leu Leu Ser Ser Tyr Gln Ser Asp Asp
20 25

<210> SEQ ID NO 19
<211> LENGTH: 26
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCR3-TM-7-1 GPCR CCR3

<400> SEQUENCE: 19

Asp Asp Leu Val Met Leu Val Thr Glu Val Ile Ala Tyr Ser His Cys
1 5 10 15

Cys Met Asn Pro Val Ile Tyr Ala Phe Val
20 25

<210> SEQ ID NO 20
<211> LENGTH: 30
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCKAR-TM-1-6 GPCR CCKAR

<400> SEQUENCE: 20

Asp Asp Glu Trp Gln Ser Ala Leu Gln Ile Leu Leu Tyr Ser Ile Ile
1 5 10 15

Phe Leu Leu Ser Val Leu Gly Asn Thr Leu Val Ile Thr Val
20 25 30

<210> SEQ ID NO 21
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCKAR-TM-2-1 GPCR CCKAR

<400> SEQUENCE: 21

Phe Leu Leu Ser Leu Ala Val Ser Asp Leu Met Leu Cys Leu Phe Cys
1 5 10 15

Met Pro Phe Asn Leu Pro
20

<210> SEQ ID NO 22
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCKAR-TM-2-2 GPCR CCKAR

-continued

<400> SEQUENCE: 22

Phe Leu Leu Ser Leu Ala Val Ser Asp Leu Met Leu Cys Leu Phe Cys
1 5 10 15

Met Pro Phe Asn Leu Ile Asp Asp
20

<210> SEQ ID NO 23
<211> LENGTH: 26
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCKAR-TM-6-4 GPCR CCKAR

<400> SEQUENCE: 23

Ile Val Val Leu Phe Phe Leu Cys Trp Met Pro Ile Phe Ser Ala Asn
1 5 10 15

Ala Trp Arg Ala Tyr Asp Thr Val Asp Asp
20 25

<210> SEQ ID NO 24
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: GPCRAelegans GPCR TM1

<400> SEQUENCE: 24

His Pro Cys Glu Asp Ile Met Gly Tyr Val Trp Leu Thr Val Val Ser
1 5 10 15

Phe Met Val Gly Ala Val Ala Leu Val Ala Asn Leu Val Val Ala Leu
20 25 30

Val Leu Leu Thr Ser Gln Arg Arg Leu Asn Val
35 40

<210> SEQ ID NO 25
<211> LENGTH: 50
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: GRH GPCR TM1

<400> SEQUENCE: 25

Asn Leu Pro Thr Leu Thr Leu Ser Gly Lys Ile Arg Val Thr Val Thr
1 5 10 15

Phe Phe Leu Phe Leu Leu Ser Ala Thr Phe Asn Ala Ser Phe Leu Leu
20 25 30

Lys Leu Gln Lys Trp Thr Gln Lys Lys Glu Lys Gly Lys Lys Leu Ser
35 40 45

Arg Met
50

<210> SEQ ID NO 26
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: TRH GPCR TM1

-continued

<400> SEQUENCE: 26

Arg Ala Val Val Ala Leu Glu Tyr Gln Val Val Thr Ile Leu Leu Val
1 5 10 15

Leu Ile Ile Cys Gly Leu Gly Ile Val Gly Asn Ile Met Val Val Leu
20 25 30

Val Val Met Arg Thr Lys His Met Arg Thr Pro
35 40

<210> SEQ ID NO 27
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: FSHprec GPCR TM1

<400> SEQUENCE: 27

Asn Pro Cys Glu Asp Ile Met Gly Tyr Asn Ile Leu Arg Val Leu Ile
1 5 10 15

Trp Phe Ile Ser Ile Leu Ala Ile Thr Gly Asn Ile Ile Val Leu Val
20 25 30

Ile Leu Thr Thr Ser Gln Tyr Lys Leu Thr Val
35 40

<210> SEQ ID NO 28
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: TSHprec GPCR TM1

<400> SEQUENCE: 28

Asn Pro Cys Glu Asp Ile Met Gly Tyr Lys Phe Leu Arg Ile Val Val
1 5 10 15

Trp Phe Val Ser Leu Leu Ala Leu Leu Gly Asn Val Phe Val Leu Leu
20 25 30

Ile Leu Leu Thr Ser His Tyr Lys Leu Asn Val
35 40

<210> SEQ ID NO 29
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: LH_CGprec GPCR TM1

<400> SEQUENCE: 29

Asn Pro Cys Glu Asp Ile Met Gly Tyr Asp Phe Leu Arg Val Leu Ile
1 5 10 15

Trp Leu Ile Asn Ile Leu Ala Ile Met Gly Asn Met Thr Val Leu Phe
20 25 30

Val Leu Leu Thr Ser Arg Tyr Lys Leu Thr Val
35 40

<210> SEQ ID NO 30
<211> LENGTH: 49
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PGE_EP1 GPCR TM1

-continued

<400> SEQUENCE: 30

Pro Asn Thr Ser Ala Val Pro Pro Ser Gly Ala Ser Pro Ala Leu Pro
1 5 10 15

Ile Phe Ser Met Thr Leu Gly Ala Val Ser Asn Leu Leu Ala Leu Ala
20 25 30

Leu Leu Ala Gln Ala Ala Gly Arg Leu Arg Arg Arg Arg Ser Ala Thr
35 40 45

Thr

<210> SEQ ID NO 31
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PGE_EP2 GPCR TM1

<400> SEQUENCE: 31

Ser Ala Ser Leu Ser Pro Asp Arg Leu Asn Ser Pro Val Thr Ile Pro
1 5 10 15

Ala Val Met Phe Ile Phe Gly Val Val Gly Asn Leu Val Ala Ile Val
20 25 30

Val Leu Cys Lys Ser Arg Lys Glu Gln Lys Glu Thr Thr
35 40 45

<210> SEQ ID NO 32
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PGE_EP3 GPCR TM1

<400> SEQUENCE: 32

Gln Trp Leu Pro Pro Gly Glu Ser Pro Ala Ile Ser Ser Val Met Phe
1 5 10 15

Ser Ala Gly Val Leu Gly Asn Leu Ile Ala Leu Ala Leu Leu Ala Arg
20 25 30

Arg Trp Arg Ser Ala Gly Arg Arg Ser Ser Leu Ser Leu
35 40 45

<210> SEQ ID NO 33
<211> LENGTH: 48
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PGF GPCR TM1

<400> SEQUENCE: 33

Ser Asn Thr Thr Cys Gln Thr Glu Asn Arg Leu Ser Val Phe Phe Ser
1 5 10 15

Val Ile Phe Met Thr Val Gly Ile Leu Ser Asn Ser Leu Ala Ile Ala
20 25 30

Ile Leu Met Lys Ala Tyr Gln Arg Phe Arg Gln Lys Ser Lys Ala Ser
35 40 45

<210> SEQ ID NO 34
<211> LENGTH: 44
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PGI GPCR TM1

-continued

<400> SEQUENCE: 34

Cys Arg Asn Leu Thr Tyr Val Arg Gly Ser Val Gly Pro Ala Thr Ser
1 5 10 15

Thr Leu Met Phe Val Ala Gly Val Val Gly Asn Gly Leu Ala Leu Gly
20 25 30

Ile Leu Ser Ala Arg Arg Pro Ala Arg Pro Ser Ala
35 40

<210> SEQ ID NO 35
<211> LENGTH: 47
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: TXA2 GPCR TM1

<400> SEQUENCE: 35

Asn Ile Thr Leu Glu Glu Arg Arg Leu Ile Ala Ser Pro Trp Phe Ala
1 5 10 15

Ala Ser Phe Cys Val Val Gly Leu Ala Ser Asn Leu Leu Ala Leu Ser
20 25 30

Val Leu Ala Gly Ala Arg Gln Gly Gly Ser His Thr Arg Ser Ser
35 40 45

<210> SEQ ID NO 36
<211> LENGTH: 46
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PAF GPCR TM1

<400> SEQUENCE: 36

His Met Asp Ser Glu Phe Arg Tyr Thr Leu Phe Pro Ile Val Tyr Ser
1 5 10 15

Ile Ile Phe Val Leu Gly Val Ile Ala Asn Gly Tyr Val Leu Trp Val
20 25 30

Phe Ala Arg Leu Tyr Pro Cys Lys Lys Phe Asn Glu Ile Lys
35 40 45

<210> SEQ ID NO 37
<211> LENGTH: 40
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: M2 GPCR TM1

<400> SEQUENCE: 37

Tyr Lys Thr Phe Glu Val Val Phe Ile Val Leu Val Ala Gly Ser Leu
1 5 10 15

Ser Leu Val Thr Ile Ile Gly Asn Ile Leu Val Met Val Ser Ile Lys
20 25 30

Val Asn Arg His Leu Gln Thr Val
35 40

<210> SEQ ID NO 38
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: M4 GPCR TM1

-continued

<400> SEQUENCE: 38

His Asn Arg Tyr Glu Thr Val Glu Met Val Phe Ile Ala Thr Val Thr
 1   5   10   15

Gly Ser Leu Ser Leu Val Thr Val Val Gly Asn Ile Leu Val Met Leu
            20   25   30

Ser Ile Lys Val Asn Arg Gln Leu Gln Thr Val
        35   40

<210> SEQ ID NO 39
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: M1 GPCR TM1

<400> SEQUENCE: 39

Gly Lys Gly Pro Trp Gln Val Ala Phe Ile Gly Ile Thr Thr Gly Leu
 1   5   10   15

Leu Ser Leu Ala Thr Val Thr Gly Asn Leu Leu Val Leu Ile Ser Phe
            20   25   30

Lys Val Asn Thr Glu Leu Lys Thr Val
        35   40

<210> SEQ ID NO 40
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: M3 GPCR TM1

<400> SEQUENCE: 40

Leu Gly Gly His Thr Val Trp Gln Val Val Phe Ile Ala Phe Leu Thr
 1   5   10   15

Gly Ile Leu Ala Leu Val Thr Ile Ile Gly Asn Ile Leu Val Ile Val
            20   25   30

Ser Phe Lys Val Asn Lys Gln Leu Lys Thr Val
        35   40

<210> SEQ ID NO 41
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: M5 GPCR TM1

<400> SEQUENCE: 41

Leu Glu Arg His Arg Leu Trp Glu Val Ile Thr Ile Ala Ala Val Thr
 1   5   10   15

Ala Val Val Ser Leu Ile Thr Ile Val Gly Asn Val Leu Val Met Ile
            20   25   30

Ser Phe Lys Val Asn Ser Gln Leu Lys Thr Val
        35   40

<210> SEQ ID NO 42
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: H1 GPCR TM1

-continued

<400> SEQUENCE: 42

Lys Thr Thr Met Ala Ser Pro Gln Leu Met Pro Leu Val Val Val Leu
1 5 10 15

Ser Thr Ile Cys Leu Val Thr Val Gly Leu Asn Leu Leu Val Leu Tyr
20 25 30

Ala Val Arg Ser Glu Arg Lys Leu His Thr Val
35 40

<210> SEQ ID NO 43
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: H2 GPCR TM1

<400> SEQUENCE: 43

Phe Cys Leu Asp Ser Thr Ala Cys Lys Ile Thr Ile Thr Val Val Leu
1 5 10 15

Ala Val Leu Ile Leu Ile Thr Val Ala Gly Asn Val Val Val Cys Leu
20 25 30

Ala Val Gly Leu Asn Arg Arg Leu Arg Asn Leu
35 40

<210> SEQ ID NO 44
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT1A GPCR TM1

<400> SEQUENCE: 44

Ile Ser Asp Val Thr Val Ser Tyr Gln Val Ile Thr Ser Leu Leu Leu
1 5 10 15

Gly Thr Leu Ile Phe Cys Ala Val Leu Gly Asn Ala Cys Val Val Ala
20 25 30

Ala Ile Ala Leu Glu Arg Ser Leu Gln Asn Val
35 40

<210> SEQ ID NO 45
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT1B GPCR TM1

<400> SEQUENCE: 45

Gln Asp Ser Ile Ser Leu Pro Trp Lys Val Leu Leu Val Met Leu Leu
1 5 10 15

Ala Leu Ile Thr Leu Ala Thr Thr Leu Ser Asn Ala Phe Val Ile Ala
20 25 30

Thr Val Tyr Arg Thr Arg Lys Leu His Thr Pro
35 40

<210> SEQ ID NO 46
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT1D GPCR TM1

-continued

<400> SEQUENCE: 46

Asp Pro Arg Thr Leu Gln Ala Leu Lys Ile Ser Leu Ala Val Val Leu
1 5 10 15

Ser Val Ile Thr Leu Ala Thr Val Leu Ser Asn Ala Phe Val Leu Thr
20 25 30

Thr Ile Leu Leu Thr Arg Lys Leu His Thr Pro
35 40

<210> SEQ ID NO 47
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT1E GPCR TM1

<400> SEQUENCE: 47

Ile Arg Pro Lys Thr Ile Thr Glu Lys Met Leu Ile Cys Met Thr Leu
1 5 10 15

Val Val Ile Thr Thr Leu Thr Thr Leu Leu Asn Leu Ala Val Ile Met
20 25 30

Ala Ile Gly Thr Thr Lys Lys Leu His Gln Pro
35 40

<210> SEQ ID NO 48
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT1F GPCR TM1

<400> SEQUENCE: 48

Glu Leu Leu Asn Arg Met Pro Ser Lys Ile Leu Val Ser Leu Thr Leu
1 5 10 15

Ser Gly Leu Ala Leu Met Thr Thr Thr Ile Asn Ser Leu Val Ile Ala
20 25 30

Ala Ile Ile Val Thr Arg Lys Leu His His Pro
35 40

<210> SEQ ID NO 49
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT2A GPCR TM1

<400> SEQUENCE: 49

Gln Glu Lys Asn Trp Ser Ala Leu Leu Thr Ala Val Val Ile Ile Leu
1 5 10 15

Thr Ile Ala Gly Asn Ile Leu Val Ile Met Ala Val Ser Leu Glu Lys
20 25 30

Lys Leu Gln Asn Ala
35

<210> SEQ ID NO 50
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT2B GPCR TM1

-continued

<400> SEQUENCE: 50

Ile Val Glu Glu Gln Gly Asn Lys Leu His Trp Ala Ala Leu Leu Ile
1 5 10 15

Leu Met Val Ile Ile Pro Thr Ile Gly Gly Asn Thr Leu Val Ile Leu
20 25 30

Ala Val Ser Leu Glu Lys Lys Leu Gln Tyr Ala
35 40

<210> SEQ ID NO 51
<211> LENGTH: 35
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT2C GPCR TM1

<400> SEQUENCE: 51

Gln Asn Trp Pro Ala Leu Ser Ile Val Ile Ile Ile Ile Met Thr Ile
1 5 10 15

Gly Gly Asn Ile Leu Val Ile Met Ala Val Ser Met Glu Lys Lys Leu
20 25 30

His Asn Ala
35

<210> SEQ ID NO 52
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT5A GPCR TM1

<400> SEQUENCE: 52

Ser Ser Pro Leu Leu Ser Val Phe Gly Val Leu Ile Leu Thr Leu Leu
1 5 10 15

Gly Phe Leu Val Ala Ala Thr Phe Ala Trp Asn Leu Leu Val Leu Ala
20 25 30

Thr Ile Leu Arg Val Arg Thr Phe His Arg Val
35 40

<210> SEQ ID NO 53
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT5Brat GPCR TM1

<400> SEQUENCE: 53

Arg Glu Pro Pro Phe Ser Ala Phe Thr Val Leu Val Val Thr Leu Leu
1 5 10 15

Val Leu Leu Ile Ala Ala Thr Phe Leu Trp Asn Leu Leu Val Leu Val
20 25 30

Thr Ile Leu Arg Val Arg Ala Phe His Arg Val
35 40

<210> SEQ ID NO 54
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT6rat GPCR TM1

-continued

<400> SEQUENCE: 54

Gly Pro Pro Pro Ala Pro Gly Gly Ser Gly Trp Val Ala Ala Ala Leu
1 5 10 15

Cys Val Val Ile Val Leu Thr Ala Ala Ala Asn Ser Leu Leu Ile Val
20 25 30

Leu Ile Cys Thr Gln Pro Ala Val Arg Asn Thr
35 40

<210> SEQ ID NO 55
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT7 GPCR TM1

<400> SEQUENCE: 55

Gln Ile Asn Tyr Gly Arg Val Glu Lys Val Val Ile Gly Ser Ile Leu
1 5 10 15

Thr Leu Ile Thr Leu Leu Thr Ile Ala Gly Asn Cys Leu Val Val Ile
20 25 30

Ser Val Cys Phe Val Lys Lys Leu Arg Gln Pro
35 40

<210> SEQ ID NO 56
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: alpha1A GPCR TM1

<400> SEQUENCE: 56

Gly Gly Leu Val Val Ser Ala Gln Gly Val Gly Val Gly Val Phe Leu
1 5 10 15

Ala Ala Phe Ile Leu Met Ala Val Ala Gly Asn Leu Leu Val Ile Leu
20 25 30

Ser Val Ala Cys Asn Arg His Leu Gln Thr Val
35 40

<210> SEQ ID NO 57
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: alpha1B GPCR TM1

<400> SEQUENCE: 57

Gln Leu Asp Ile Thr Arg Ala Ile Ser Val Gly Leu Val Leu Gly Ala
1 5 10 15

Phe Ile Leu Phe Ala Ile Val Gly Asn Ile Leu Val Ile Leu Ser Val
20 25 30

Ala Cys Asn Arg His Leu Arg Thr Pro
35 40

<210> SEQ ID NO 58
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: alpha1C GPCR TM1

-continued

<400> SEQUENCE: 58

Pro Ala Pro Val Asn Ile Ser Lys Ala Ile Leu Leu Gly Val Ile Leu
1 5 10 15

Gly Gly Leu Ile Leu Phe Gly Val Leu Cys Asn Ile Leu Val Ile Leu
20 25 30

Ser Val Ala Cys His Arg His Leu His Ser Val
35 40

<210> SEQ ID NO 59
<211> LENGTH: 40
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: alpha2A GPCR TM1

<400> SEQUENCE: 59

Tyr Ser Leu Gln Val Thr Leu Thr Leu Val Cys Leu Ala Gly Leu Leu
1 5 10 15

Met Leu Leu Thr Val Phe Gly Asn Val Leu Val Ile Ile Ala Val Phe
20 25 30

Thr Ser Arg Ala Leu Lys Ala Pro
35 40

<210> SEQ ID NO 60
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: alpha2B GPCR TM1

<400> SEQUENCE: 60

Gln Asp Pro Tyr Ser Val Gln Ala Thr Ala Ala Ile Ala Ala Ala Ile
1 5 10 15

Thr Phe Leu Ile Leu Phe Thr Ile Phe Gly Asn Ala Leu Val Ile Leu
20 25 30

Ala Val Leu Thr Ser Arg Ser Leu Arg Ala Pro
35 40

<210> SEQ ID NO 61
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: alpha2C1 and alpha2C2 GPCR TM1

<400> SEQUENCE: 61

Arg Gly Gln Tyr Ser Ala Gly Ala Val Ala Gly Leu Ala Ala Val Val
1 5 10 15

Gly Phe Leu Ile Val Phe Thr Val Val Gly Asn Val Leu Val Val Ile
20 25 30

Ala Val Leu Thr Ser Arg Ala Leu Arg Ala Pro
35 40

<210> SEQ ID NO 62
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: beta1 GPCR TM1

-continued

<400> SEQUENCE: 62

Glu Pro Leu Ser Gln Gln Trp Thr Ala Gly Met Gly Leu Leu Met Ala
1 5 10 15

Leu Ile Val Leu Leu Ile Val Ala Gly Asn Val Leu Val Ile Val Ala
20 25 30

Ile Ala Lys Thr Pro Arg Leu Gln Thr Leu
35 40

<210> SEQ ID NO 63
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: beta2 GPCR TM1

<400> SEQUENCE: 63

Gln Gln Arg Asp Glu Val Trp Val Val Gly Met Gly Ile Val Met Ser
1 5 10 15

Leu Ile Val Leu Ala Ile Val Phe Gly Asn Val Leu Val Ile Thr Ala
20 25 30

Ile Ala Lys Phe Glu Arg Leu Gln Thr Val
35 40

<210> SEQ ID NO 64
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: beta3 GPCR TM1

<400> SEQUENCE: 64

Gly Leu Pro Gly Val Pro Trp Glu Ala Ala Leu Ala Gly Ala Leu Leu
1 5 10 15

Ala Leu Ala Val Leu Ala Thr Val Gly Gly Asn Leu Leu Val Ile Val
20 25 30

Ala Ile Ala Trp Thr Pro Arg Leu Gln Thr Met
35 40

<210> SEQ ID NO 65
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: beta4turkey GPCR TM1

<400> SEQUENCE: 65

Ser Trp Ala Ala Val Leu Ser Arg Gln Trp Ala Val Gly Ala Ala Leu
1 5 10 15

Ser Ile Thr Ile Leu Val Ile Val Ala Gly Asn Leu Leu Val Ile Val
20 25 30

Ala Ile Ala Lys Thr Pro Arg Leu Gln Thr Met
35 40

<210> SEQ ID NO 66
<211> LENGTH: 44
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: D1A GPCR TM1

-continued

<400> SEQUENCE: 66

Val Val Glu Arg Asp Phe Ser Val Arg Ile Leu Thr Ala Cys Phe Leu
1 5 10 15

Ser Leu Leu Ile Leu Ser Thr Leu Leu Gly Asn Thr Leu Val Cys Ala
20 25 30

Ala Val Ile Arg Phe Arg His Leu Arg Ser Lys Val
35 40

<210> SEQ ID NO 67
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: D2 GPCR TM1

<400> SEQUENCE: 67

Asp Gly Lys Ala Asp Arg Pro His Tyr Asn Tyr Tyr Ala Thr Leu Leu
1 5 10 15

Thr Leu Leu Ile Ala Val Ile Val Phe Gly Asn Val Leu Val Cys Met
20 25 30

Ala Val Ser Arg Glu Lys Ala Leu Gln Thr Thr
35 40

<210> SEQ ID NO 68
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: D3 GPCR TM1

<400> SEQUENCE: 68

Thr Gly Ala Ser Gln Ala Arg Pro His Ala Tyr Tyr Ala Leu Ser Tyr
1 5 10 15

Cys Ala Leu Ile Leu Ala Ile Val Phe Gly Asn Gly Leu Val Cys Met
20 25 30

Ala Val Leu Lys Glu Arg Ala Leu Gln Thr Thr
35 40

<210> SEQ ID NO 69
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: D4 GPCR TM1

<400> SEQUENCE: 69

Ala Ser Ala Gly Leu Ala Gly Gln Gly Ala Ala Ala Leu Val Gly Gly
1 5 10 15

Val Leu Leu Ile Gly Ala Val Leu Ala Gly Asn Ser Leu Val Cys Val
20 25 30

Ser Val Ala Thr Glu Arg Ala Leu Gln Thr Pro
35 40

<210> SEQ ID NO 70
<211> LENGTH: 44
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: D5 GPCR TM1

-continued

<400> SEQUENCE: 70

Gly Ala Pro Pro Leu Gly Pro Ser Gln Val Val Thr Ala Cys Leu Leu
1 5 10 15

Thr Leu Leu Ile Ile Trp Thr Leu Leu Gly Asn Val Leu Val Cys Ala
20 25 30

Ala Ile Val Arg Ser Arg His Leu Arg Ala Asn Met
35 40

<210> SEQ ID NO 71
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: A1 GPCR TM1

<400> SEQUENCE: 71

Met Pro Pro Ser Ile Ser Ala Phe Gln Ala Ala Tyr Ile Gly Ile Glu
1 5 10 15

Val Leu Ile Ala Leu Val Ser Val Pro Gly Asn Val Leu Val Ile Trp
20 25 30

Ala Val Lys Val Asn Gln Ala Leu Arg Asp Ala
35 40

<210> SEQ ID NO 72
<211> LENGTH: 40
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: A2a GPCR TM1

<400> SEQUENCE: 72

Met Pro Ile Met Gly Ser Ser Val Tyr Ile Thr Val Glu Leu Ala Ile
1 5 10 15

Ala Val Leu Ala Ile Leu Gly Asn Val Leu Val Cys Trp Ala Val Trp
20 25 30

Leu Asn Ser Asn Leu Gln Asn Val
35 40

<210> SEQ ID NO 73
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: A2b GPCR TM1

<400> SEQUENCE: 73

Met Leu Leu Glu Thr Gln Asp Ala Leu Tyr Val Ala Leu Glu Leu Val
1 5 10 15

Ile Ala Ala Leu Ser Val Ala Gly Asn Val Leu Val Cys Ala Ala Val
20 25 30

Gly Thr Ala Asn Thr Leu Gln Thr Pro
35 40

<210> SEQ ID NO 74
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: A3 GPCR TM1

-continued

<400> SEQUENCE: 74

Asn Ser Thr Thr Leu Ser Leu Ala Asn Val Thr Tyr Ile Thr Met Glu
1 5 10 15

Ile Phe Ile Gly Leu Cys Ala Ile Val Gly Asn Val Leu Val Ile Cys
20 25 30

Val Val Lys Leu Asn Pro Ser Leu Gln Thr Thr
35 40

<210> SEQ ID NO 75
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OCdrome GPCR TM1

<400> SEQUENCE: 75

Leu Ala Val Pro Glu Trp Glu Ala Leu Leu Thr Ala Leu Val Leu Ser
1 5 10 15

Val Ile Ile Val Leu Thr Ile Ile Gly Asn Ile Leu Val Ile Leu Ser
20 25 30

Val Phe Thr Tyr Lys Pro Leu Arg Ile Val
35 40

<210> SEQ ID NO 76
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: ACTH GPCR TM1

<400> SEQUENCE: 76

Arg Asn Asn Ser Asp Cys Pro Arg Val Val Leu Pro Glu Glu Ile Phe
1 5 10 15

Phe Thr Ile Ser Ile Val Gly Val Leu Glu Asn Leu Ile Val Leu Leu
20 25 30

Ala Val Phe Lys Asn Lys Asn Leu Gln Ala Pro
35 40

<210> SEQ ID NO 77
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: MSH GPCR TM1

<400> SEQUENCE: 77

Gln Thr Gly Ala Arg Cys Leu Glu Val Ser Ile Ser Asp Gly Leu Phe
1 5 10 15

Leu Ser Leu Gly Leu Val Ser Leu Val Glu Asn Ala Leu Val Val Ala
20 25 30

Thr Ile Ala Lys Asn Arg Asn Leu His Ser Pro
35 40

<210> SEQ ID NO 78
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: MC3 GPCR TM1

-continued

<400> SEQUENCE: 78

Ser Ser Ser Ala Phe Cys Glu Gln Val Phe Ile Lys Pro Glu Ile Phe
1 5 10 15

Leu Ser Leu Gly Ile Val Ser Leu Leu Glu Asn Ile Leu Val Ile Leu
20 25 30

Ala Val Val Arg Asn Gly Asn Leu His Ser Pro
35 40

<210> SEQ ID NO 79
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: MC4 GPCR TM1

<400> SEQUENCE: 79

Ser Asp Gly Gly Cys Tyr Glu Gln Leu Phe Val Ser Pro Glu Val Phe
1 5 10 15

Val Thr Leu Gly Val Ile Ser Leu Leu Glu Asn Ile Leu Val Ile Val
20 25 30

Ala Ile Ala Lys Asn Lys Asn Leu His Ser Pro
35 40

<210> SEQ ID NO 80
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: MC5 GPCR TM1

<400> SEQUENCE: 80

Asn Lys Ser Ser Pro Cys Glu Asp Met Gly Ile Ala Val Glu Val Phe
1 5 10 15

Leu Thr Leu Gly Val Ile Ser Leu Leu Glu Asn Ile Leu Val Ile Gly
20 25 30

Ala Ile Val Lys Asn Lys Asn Leu His Ser Pro
35 40

<210> SEQ ID NO 81
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: melatonin GPCR TM1

<400> SEQUENCE: 81

Asp Gly Ala Arg Pro Ser Trp Leu Ala Ser Ala Leu Ala Cys Val Leu
1 5 10 15

Ile Phe Thr Ile Val Val Asp Ile Leu Gly Asn Leu Leu Val Ile Leu
20 25 30

Ser Val Tyr Arg Asn Lys Lys Leu Arg Asn Ala
35 40

<210> SEQ ID NO 82
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: oxytocin GPCR TM1

-continued

<400> SEQUENCE: 82

Arg Arg Asn Glu Ala Leu Ala Arg Val Glu Val Ala Val Leu Cys Leu
1 5 10 15

Ile Leu Leu Leu Ala Leu Ser Gly Asn Ala Cys Val Leu Leu Ala Leu
20 25 30

Arg Thr Thr Arg Gln Lys His Ser Arg
35 40

<210> SEQ ID NO 83
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: conopressinLs GPCR TM1

<400> SEQUENCE: 83

Phe His Gly Val Asp Glu Asp Leu Leu Lys Ile Glu Ile Ala Val Gln
1 5 10 15

Ala Thr Ile Leu Tyr Met Thr Leu Phe Gly Asn Gly Ile Val Leu Leu
20 25 30

Val Leu Arg Leu Arg Arg Gln Lys Leu Thr Arg
35 40

<210> SEQ ID NO 84
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: V1A GPCR TM1

<400> SEQUENCE: 84

Arg Asp Val Arg Asn Glu Glu Leu Ala Lys Leu Glu Ile Ala Val Leu
1 5 10 15

Ala Val Thr Phe Ala Val Ala Val Leu Gly Asn Ser Ser Val Leu Leu
20 25 30

Ala Leu His Arg Thr Pro Arg Lys Thr Ser Arg
35 40

<210> SEQ ID NO 85
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: V1B GPCR TM1

<400> SEQUENCE: 85

Trp Leu Gly Arg Asp Glu Glu Leu Ala Lys Val Glu Ile Gly Val Leu
1 5 10 15

Ala Thr Val Leu Val Leu Ala Thr Gly Gly Asn Leu Ala Val Leu Leu
20 25 30

Thr Leu Gly Gln Leu Gly Arg Lys Arg Ser Arg
35 40

<210> SEQ ID NO 86
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: V2 GPCR TM1

-continued

<400> SEQUENCE: 86

Leu Asp Thr Arg Asp Pro Leu Leu Ala Arg Ala Glu Leu Ala Leu Leu
1 5 10 15

Ser Ile Val Phe Val Ala Val Ala Leu Ser Asn Gly Leu Val Leu Ala
20 25 30

Ala Leu Ala Arg Arg Gly Arg Arg Gly His Trp Ala Pro
35 40 45

<210> SEQ ID NO 87
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCK_A GPCR TM1

<400> SEQUENCE: 87

Pro Arg Pro Ser Lys Glu Trp Gln Pro Ala Val Gln Ile Leu Leu Tyr
1 5 10 15

Ser Leu Ile Phe Leu Leu Ser Val Leu Gly Asn Thr Leu Val Ile Thr
20 25 30

Val Leu Ile Arg Asn Lys Arg Met Arg Thr Val
35 40

<210> SEQ ID NO 88
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCK_B GPCR TM1

<400> SEQUENCE: 88

Gly Ala Gly Thr Arg Glu Leu Glu Leu Ala Ile Arg Ile Thr Leu Tyr
1 5 10 15

Ala Val Ile Phe Leu Met Ser Val Gly Gly Asn Met Leu Ile Ile Val
20 25 30

Val Leu Gly Leu Ser Arg Arg Leu Arg Thr Val
35 40

<210> SEQ ID NO 89
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: NPY1 GPCR TM1

<400> SEQUENCE: 89

Asp Cys His Leu Pro Leu Ala Met Ile Phe Thr Leu Ala Leu Ala Tyr
1 5 10 15

Gly Ala Val Ile Ile Leu Gly Val Ser Gly Asn Leu Ala Leu Ile Ile
20 25 30

Ile Ile Leu Lys Gln Lys Glu Met Arg Asn Val
35 40

<210> SEQ ID NO 90
<211> LENGTH: 46
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: NTR GPCR TM1

-continued

<400> SEQUENCE: 90

Asp Val Asn Thr Asp Ile Tyr Ser Lys Val Leu Val Thr Ala Val Tyr
1 5 10 15

Leu Ala Leu Phe Val Val Gly Thr Val Gly Asn Thr Val Thr Ala Phe
20 25 30

Thr Leu Ala Arg Lys Lys Ser Leu Gln Ser Leu Gln Ser Thr
35 40 45

<210> SEQ ID NO 91
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: NK1 GPCR TM1

<400> SEQUENCE: 91

Gln Phe Val Gln Pro Ala Trp Gln Ile Val Leu Trp Ala Ala Ala Tyr
1 5 10 15

Thr Val Ile Val Val Thr Ser Val Val Gly Asn Val Val Val Met Trp
20 25 30

Ile Ile Leu Ala His Lys Arg Met Arg Thr Val
35 40

<210> SEQ ID NO 92
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: NK2 GPCR TM1

<400> SEQUENCE: 92

Ala Phe Ser Met Pro Ser Trp Gln Leu Ala Leu Trp Ala Pro Ala Tyr
1 5 10 15

Leu Ala Leu Val Leu Val Ala Val Thr Gly Asn Ala Ile Val Ile Trp
20 25 30

Ile Ile Leu Ala His Arg Arg Met Arg Thr Val
35 40

<210> SEQ ID NO 93
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: NK3 GPCR TM1

<400> SEQUENCE: 93

Gln Phe Val Gln Pro Ser Trp Arg Ile Ala Leu Trp Ser Leu Ala Tyr
1 5 10 15

Gly Val Val Val Ala Val Ala Val Leu Gly Asn Leu Ile Val Ile Trp
20 25 30

Ile Ile Leu Ala His Lys Arg Met Arg Thr Val
35 40

<210> SEQ ID NO 94
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: blueops GPCR TM1

-continued

<400> SEQUENCE: 94

Tyr His Ile Ala Pro Val Trp Ala Phe Tyr Leu Gln Ala Ala Phe Met
1 5 10 15

Gly Thr Val Phe Leu Ile Gly Phe Pro Leu Asn Ala Met Val Leu Val
20 25 30

Ala Thr Leu Arg Tyr Lys Lys Leu Arg Gln Pro
35 40

<210> SEQ ID NO 95
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: greenops GPCR TM1

<400> SEQUENCE: 95

Tyr His Ile Ala Pro Arg Trp Val Tyr His Leu Thr Ser Val Trp Met
1 5 10 15

Ile Phe Val Val Ile Ala Ser Val Phe Thr Asn Gly Leu Val Leu Ala
20 25 30

Ala Thr Met Lys Phe Lys Lys Leu Arg His Pro
35 40

<210> SEQ ID NO 96
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: redops GPCR TM1

<400> SEQUENCE: 96

Tyr His Ile Ala Pro Arg Trp Val Tyr His Leu Thr Ser Val Trp Met
1 5 10 15

Ile Phe Val Val Thr Ala Ser Val Phe Thr Asn Gly Leu Val Leu Ala
20 25 30

Ala Thr Met Lys Phe Lys Lys Leu Arg His Pro
35 40

<210> SEQ ID NO 97
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: rhodopsin GPCR TM1

<400> SEQUENCE: 97

Tyr Tyr Leu Ala Glu Pro Trp Gln Phe Ser Met Leu Ala Ala Tyr Met
1 5 10 15

Phe Leu Leu Ile Val Leu Gly Phe Pro Ile Asn Phe Leu Thr Leu Tyr
20 25 30

Val Thr Val Gln His Lys Lys Leu Arg Thr Pro
35 40

<210> SEQ ID NO 98
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: violetopsGg GPCR TM1

-continued

<400> SEQUENCE: 98

Tyr His Ile Ala Pro Pro Trp Ala Phe Tyr Leu Gln Thr Ala Phe Met
1 5 10 15

Gly Ile Val Phe Ala Val Gly Thr Pro Leu Asn Ala Val Val Leu Trp
20 25 30

Val Thr Val Arg Tyr Lys Arg Leu Arg Gln Pro
35 40

<210> SEQ ID NO 99
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: opsin_crab GPCR TM1

<400> SEQUENCE: 99

Phe Pro Pro Met Asn Pro Leu Trp Tyr Ser Ile Leu Gly Val Ala Met
1 5 10 15

Ile Ile Leu Gly Ile Ile Cys Val Leu Gly Asn Gly Met Val Ile Tyr
20 25 30

Leu Met Met Thr Thr Lys Ser Leu Arg Thr Pro
35 40

<210> SEQ ID NO 100
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: ET_Aprec GPCR TM1

<400> SEQUENCE: 100

Gln Thr Lys Ile Thr Ser Ala Phe Lys Tyr Ile Asn Thr Val Ile Ser
1 5 10 15

Cys Thr Ile Phe Ile Val Gly Met Val Gly Asn Ala Thr Leu Leu Arg
20 25 30

Ile Ile Tyr Gln Asn Lys Cys Met Arg Asn Gly
35 40

<210> SEQ ID NO 101
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: ET_Bprec GPCR TM1

<400> SEQUENCE: 101

Pro Ile Glu Ile Lys Glu Thr Phe Lys Tyr Ile Asn Thr Val Val Ser
1 5 10 15

Cys Leu Val Phe Val Leu Gly Ile Ile Gly Asn Ser Thr Leu Leu Arg
20 25 30

Ile Ile Tyr Lys Asn Lys Cys Met Arg Asn Gly
35 40

<210> SEQ ID NO 102
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: ET_Cfrog GPCR TM1

-continued

<400> SEQUENCE: 102

Arg Ala Lys Ile Arg His Ala Phe Lys Tyr Val Thr Thr Ile Leu Ser
1 5 10 15

Cys Val Ile Phe Leu Val Gly Ile Val Gly Asn Ser Thr Leu Leu Arg
20 25 30

Ile Ile Tyr Lys Asn Lys Cys Met Arg Asn Gly
35 40

<210> SEQ ID NO 103
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: galanin GPCR TM1

<400> SEQUENCE: 103

Pro Leu Phe Gly Ile Gly Val Glu Asn Phe Val Thr Leu Val Val Phe
1 5 10 15

Gly Leu Ile Phe Ala Leu Gly Val Leu Gly Asn Ser Leu Val Ile Thr
20 25 30

Val Leu Ala Arg Ser Lys Pro Gly Lys Pro Arg Ser Thr
35 40 45

<210> SEQ ID NO 104
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: NMB GPCR TM1

<400> SEQUENCE: 104

Gly Thr Thr Thr Glu Leu Val Ile Arg Cys Val Ile Pro Ser Leu Tyr
1 5 10 15

Leu Leu Ile Ile Thr Val Gly Leu Leu Gly Asn Ile Met Leu Val Lys
20 25 30

Ile Phe Ile Thr Asn Ser Ala Met Arg Ser Val
35 40

<210> SEQ ID NO 105
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: GRP GPCR TM1

<400> SEQUENCE: 105

Asp Asp Trp Ser His Pro Gly Ile Leu Tyr Val Ile Pro Ala Val Tyr
1 5 10 15

Gly Val Ile Ile Leu Ile Gly Leu Ile Gly Asn Ile Thr Leu Ile Lys
20 25 30

Ile Phe Cys Thr Val Lys Ser Met Arg Asn Val
35 40

<210> SEQ ID NO 106
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: BRS3 GPCR TM1

-continued

<400> SEQUENCE: 106

Asp Asn Ser Pro Gly Ile Glu Ala Leu Cys Ala Ile Tyr Ile Thr Tyr
1 5 10 15

Ala Val Ile Ile Ser Val Gly Ile Leu Gly Asn Ala Ile Leu Ile Lys
20 25 30

Val Phe Phe Lys Thr Lys Ser Met Gln Thr Val
35 40

<210> SEQ ID NO 107
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: deltaOP GPCR TM1

<400> SEQUENCE: 107

Gly Ser Ala Ser Ser Leu Ala Leu Ala Ile Ala Ile Thr Ala Leu Tyr
1 5 10 15

Ser Ala Val Cys Ala Val Gly Leu Leu Gly Asn Val Leu Val Met Phe
20 25 30

Gly Ile Val Arg Tyr Thr Lys Met Lys Thr Ala
35 40

<210> SEQ ID NO 108
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: kappaOP GPCR TM1

<400> SEQUENCE: 108

Pro Ala His Ile Ser Pro Ala Ile Pro Val Ile Ile Thr Ala Val Tyr
1 5 10 15

Ser Val Val Phe Val Val Gly Leu Val Gly Asn Ser Leu Val Met Phe
20 25 30

Val Ile Ile Arg Tyr Thr Lys Met Lys Thr Ala
35 40

<210> SEQ ID NO 109
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: muOP GPCR TM1

<400> SEQUENCE: 109

Gly Ser Pro Ser Met Ile Thr Ala Ile Thr Ile Met Ala Leu Tyr Ser
1 5 10 15

Ile Val Cys Val Val Gly Leu Phe Gly Asn Phe Leu Val Met Tyr Val
20 25 30

Ile Val Arg Tyr Thr Lys Met Lys Thr Ala
35 40

<210> SEQ ID NO 110
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OPRX GPCR TM1

-continued

<400> SEQUENCE: 110

Gly Ala Phe Leu Pro Leu Gly Leu Lys Val Thr Ile Val Gly Leu Tyr
1 5 10 15

Leu Ala Val Cys Val Gly Gly Leu Leu Gly Asn Cys Leu Val Met Tyr
20 25 30

Val Ile Leu Arg His Thr Lys Met Lys Thr Ala
35 40

<210> SEQ ID NO 111
<211> LENGTH: 44
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CB1 GPCR TM1

<400> SEQUENCE: 111

Phe Met Val Leu Asn Pro Ser Gln Gln Leu Ala Ile Ala Val Leu Ser
1 5 10 15

Leu Thr Leu Gly Thr Phe Thr Val Leu Glu Asn Leu Leu Val Leu Cys
20 25 30

Val Ile Leu His Ser Arg Ser Leu Arg Cys Arg Pro
35 40

<210> SEQ ID NO 112
<211> LENGTH: 44
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CB2 GPCR TM1

<400> SEQUENCE: 112

Tyr Met Ile Leu Ser Gly Pro Gln Lys Thr Ala Val Ala Val Leu Cys
1 5 10 15

Thr Leu Leu Gly Leu Leu Ser Ala Leu Glu Asn Val Ala Val Leu Tyr
20 25 30

Leu Ile Leu Ser Ser His Gln Leu Arg Arg Lys Pro
35 40

<210> SEQ ID NO 113
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: SSTR1 GPCR TM1

<400> SEQUENCE: 113

Thr Leu Ser Glu Gly Gln Gly Ser Ala Ile Leu Ile Ser Phe Ile Tyr
1 5 10 15

Ser Val Val Cys Leu Val Gly Leu Cys Gly Asn Ser Met Val Ile Tyr
20 25 30

Val Ile Leu Arg Tyr Ala Lys Met Lys Thr Ala
35 40

<210> SEQ ID NO 114
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: SSTR2 GPCR TM1

-continued

<400> SEQUENCE: 114

Glu Pro Tyr Tyr Asp Leu Thr Ser Asn Ala Val Leu Thr Phe Ile Tyr
1 5 10 15

Phe Val Val Cys Ile Ile Gly Leu Cys Gly Asn Thr Leu Val Ile Tyr
20 25 30

Val Ile Leu Arg Tyr Ala Lys Met Lys Thr Ile
35 40

<210> SEQ ID NO 115
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: SSTR3 GPCR TM1

<400> SEQUENCE: 115

Ser Pro Ala Gly Leu Ala Val Ser Gly Val Leu Ile Pro Leu Val Tyr
1 5 10 15

Leu Val Val Cys Val Val Gly Leu Leu Gly Asn Ser Leu Val Ile Tyr
20 25 30

Val Val Leu Arg His Thr Ala Ser Pro Ser Val
35 40

<210> SEQ ID NO 116
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: SSTR4 GPCR TM1

<400> SEQUENCE: 116

Gly Asp Ala Arg Ala Ala Gly Met Val Ala Ile Gln Cys Ile Tyr Ala
1 5 10 15

Leu Val Cys Leu Val Gly Leu Val Gly Asn Ala Leu Val Ile Phe Val
20 25 30

Ile Leu Arg Tyr Ala Lys Met Lys Thr Ala
35 40

<210> SEQ ID NO 117
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: SSTR5 GPCR TM1

<400> SEQUENCE: 117

Pro Ala Pro Ser Ala Gly Ala Arg Ala Val Leu Val Pro Val Leu Tyr
1 5 10 15

Leu Leu Val Cys Ala Ala Gly Leu Gly Gly Asn Thr Leu Val Ile Tyr
20 25 30

Val Val Leu Arg Phe Ala Lys Met Lys Thr Val
35 40

<210> SEQ ID NO 118
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: IL8A GPCR TM1

-continued

<400> SEQUENCE: 118

Met Leu Glu Thr Glu Thr Leu Asn Lys Tyr Val Val Ile Ile Ala Tyr
1 5 10 15

Ala Leu Val Phe Leu Leu Ser Leu Leu Gly Asn Ser Leu Val Met Leu
20 25 30

Val Ile Leu Tyr Ser Arg Val Gly Arg Ser Val
35 40

<210> SEQ ID NO 119
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: IL8B GPCR TM1

<400> SEQUENCE: 119

Glu Pro Glu Ser Leu Glu Ile Asn Lys Tyr Phe Val Val Ile Ile Tyr
1 5 10 15

Ala Leu Val Phe Leu Leu Ser Leu Leu Gly Asn Ser Leu Val Met Leu
20 25 30

Val Ile Leu Tyr Ser Arg Val Gly Arg Ser Val
35 40

<210> SEQ ID NO 120
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AT1 AND AT1brat GPCR TM1

<400> SEQUENCE: 120

Lys Ala Gly Arg His Asn Tyr Ile Phe Val Met Ile Pro Thr Leu Tyr
1 5 10 15

Ser Ile Ile Phe Val Val Gly Ile Phe Gly Asn Ser Leu Val Val Ile
20 25 30

Val Ile Tyr Phe Tyr Met Lys Leu Lys Thr Val
35 40

<210> SEQ ID NO 121
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AT2 GPCR TM1

<400> SEQUENCE: 121

Gln Lys Pro Ser Asp Lys His Leu Asp Ala Ile Pro Ile Leu Tyr Tyr
1 5 10 15

Ile Ile Phe Val Ile Gly Phe Leu Val Asn Ile Val Val Val Thr Leu
20 25 30

Phe Cys Cys Gln Lys Gly Pro Lys Lys Val
35 40

<210> SEQ ID NO 122
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: BK1 GPCR TM1

-continued

<400> SEQUENCE: 122

Ala Pro Glu Ala Trp Asp Leu Leu His Arg Val Leu Pro Thr Phe Ile
1 5 10 15

Ile Ser Ile Cys Phe Phe Gly Leu Leu Gly Asn Leu Phe Val Leu Leu
20 25 30

Val Phe Leu Leu Pro Arg Arg Gln Leu Asn Val
35 40

<210> SEQ ID NO 123
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: BK2 GPCR TM1

<400> SEQUENCE: 123

Gln Val Glu Trp Leu Gly Trp Leu Asn Thr Ile Gln Pro Pro Phe Leu
1 5 10 15

Trp Val Leu Phe Val Leu Ala Thr Leu Glu Asn Ile Phe Val Leu Ser
20 25 30

Val Phe Cys Leu His Lys Ser Ser Cys Thr Val
35 40

<210> SEQ ID NO 124
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P2Y7 GPCR TM1

<400> SEQUENCE: 124

Pro Ser Leu Gly Val Glu Phe Ile Ser Leu Leu Ala Ile Ile Leu Leu
1 5 10 15

Ser Val Ala Leu Ala Val Gly Leu Pro Gly Asn Ser Phe Val Val Trp
20 25 30

Ser Ile Leu Lys Arg Met Gln Lys Arg Ser Val
35 40

<210> SEQ ID NO 125
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P2Y6 GPCR TM1

<400> SEQUENCE: 125

Cys Val Tyr Arg Glu Asp Phe Lys Arg Leu Leu Leu Pro Pro Val Tyr
1 5 10 15

Ser Val Val Leu Val Val Gly Leu Pro Leu Asn Val Cys Val Ile Ala
20 25 30

Gln Ile Cys Ala Ser Arg Arg Thr Leu Thr Arg
35 40

<210> SEQ ID NO 126
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P2Y5 GPCR TM1

-continued

<400> SEQUENCE: 126

Cys Ser Thr Glu Asp Ser Phe Lys Tyr Thr Leu Tyr Gly Cys Val Phe
1 5 10 15

Ser Met Val Phe Val Leu Gly Leu Ile Ala Asn Cys Val Ala Ile Tyr
20 25 30

Ile Phe Thr Phe Thr Leu Lys Val Arg Asn Glu
35 40

<210> SEQ ID NO 127
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P2Y4 GPCR TM1

<400> SEQUENCE: 127

Cys Trp Phe Asp Glu Asp Phe Lys Phe Ile Leu Leu Pro Val Ser Tyr
1 5 10 15

Ala Val Val Phe Val Leu Gly Leu Gly Leu Asn Ala Pro Thr Leu Trp
20 25 30

Leu Phe Ile Phe Arg Leu Arg Pro Trp Asp Ala
35 40

<210> SEQ ID NO 128
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P2Y3chick GPCR TM1

<400> SEQUENCE: 128

Cys Thr Phe His Glu Glu Phe Lys Gln Val Leu Leu Pro Leu Val Tyr
1 5 10 15

Ser Val Val Phe Leu Leu Gly Leu Pro Leu Asn Ala Val Val Ile Gly
20 25 30

Gln Ile Trp Leu Ala Arg Lys Ala Leu Thr Arg
35 40

<210> SEQ ID NO 129
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P2Y2 GPCR TM1

<400> SEQUENCE: 129

Cys Arg Phe Asn Glu Asp Phe Lys Tyr Val Leu Leu Pro Val Ser Tyr
1 5 10 15

Gly Val Val Cys Val Leu Gly Leu Cys Leu Asn Ala Val Gly Leu Tyr
20 25 30

Ile Phe Leu Cys Arg Leu Lys Thr Trp Asn Ala
35 40

<210> SEQ ID NO 130
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P2Y1 GPCR TM1

-continued

<400> SEQUENCE: 130

Ala Leu Thr Lys Thr Gly Phe Gln Phe Tyr Tyr Leu Pro Ala Val Tyr
1 5 10 15

Ile Leu Val Phe Ile Ile Gly Phe Leu Gly Asn Ser Val Ala Ile Trp
20 25 30

Met Phe Val Phe His Met Lys Pro Trp Ser Gly
35 40

<210> SEQ ID NO 131
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: THRprec GPCR TM1

<400> SEQUENCE: 131

Gly Tyr Leu Thr Ser Ser Trp Leu Thr Leu Phe Val Pro Ser Val Tyr
1 5 10 15

Thr Gly Val Phe Val Val Ser Leu Pro Leu Asn Ile Met Ala Ile Val
20 25 30

Val Phe Ile Leu Lys Met Lys Val Lys Lys Pro
35 40

<210> SEQ ID NO 132
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: C5a GPCR TM1

<400> SEQUENCE: 132

Thr Ser Asn Thr Leu Arg Val Pro Asp Ile Leu Ala Leu Val Ile Phe
1 5 10 15

Ala Val Val Phe Leu Val Gly Val Leu Gly Asn Ala Leu Val Val Trp
20 25 30

Val Thr Ala Phe Glu Ala Lys Arg Thr Ile
35 40

<210> SEQ ID NO 133
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: GP01mouse GPCR TM1

<400> SEQUENCE: 133

Ala Glu Ser Glu Pro Glu Leu Val Val Asn Pro Trp Asp Ile Val Leu
1 5 10 15

Cys Ser Ser Gly Thr Leu Ile Cys Cys Glu Asn Ala Val Val Val Leu
20 25 30

Ile Ile Phe His Ser Pro Ser Leu Arg Ala Pro
35 40

<210> SEQ ID NO 134
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: R334rat GPCR TM1

-continued

<400> SEQUENCE: 134

Val Glu Ser Glu Pro Glu Leu Val Val Asn Pro Trp Asp Ile Val Leu
1 5 10 15

Cys Ser Ser Gly Thr Leu Ile Cys Cys Glu Asn Ala Val Val Val Leu
20 25 30

Ile Ile Phe His Ser Pro Ser Leu Arg Ala Pro
35 40

<210> SEQ ID NO 135
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: GP21mouse GPCR TM1

<400> SEQUENCE: 135

Gly Pro Ala Thr Leu Leu Pro Ser Pro Arg Ala Trp Asp Val Val Leu
1 5 10 15

Cys Ile Ser Gly Thr Leu Val Ser Cys Glu Asn Ala Leu Val Val Ala
20 25 30

Ile Ile Val Gly Thr Pro Ala Phe Arg Ala Pro
35 40

<210> SEQ ID NO 136
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: GCRCmouse GPCR TM1

<400> SEQUENCE: 136

Ala Glu Ser Gln Asn Pro Thr Val Lys Ala Leu Leu Ile Val Ala Tyr
1 5 10 15

Ser Phe Thr Ile Val Phe Ser Leu Phe Gly Asn Val Leu Val Cys His
20 25 30

Val Ile Phe Lys Asn Gln Arg Met His Ser Ala
35 40

<210> SEQ ID NO 137
<211> LENGTH: 40
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: TXKR GPCR TM1

<400> SEQUENCE: 137

Gln Pro Pro Trp Ala Val Ala Leu Trp Ser Leu Ala Tyr Gly Ala Val
1 5 10 15

Val Ala Val Ala Val Leu Gly Asn Leu Val Val Ile Trp Ile Val Leu
20 25 30

Ala His Lys Arg Met Arg Thr Val
35 40

<210> SEQ ID NO 138
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: G10Drat GPCR TM1

-continued

<400> SEQUENCE: 138

Met Glu Leu Asn Glu Asn Thr Lys Gln Val Val Leu Phe Val Phe Tyr
1 5 10 15

Leu Ala Ile Phe Val Val Gly Leu Val Glu Asn Val Leu Val Ile Cys
20 25 30

Val Asn Cys Arg Arg Ser Gly Arg Val Gly Met
35 40

<210> SEQ ID NO 139
<211> LENGTH: 44
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: RDC1 GPCR TM1

<400> SEQUENCE: 139

Asn Met Pro Asn Lys Ser Val Leu Leu Tyr Thr Leu Ser Phe Ile Tyr
1 5 10 15

Ile Phe Ile Phe Val Ile Gly Met Ile Ala Asn Ser Val Val Val Trp
20 25 30

Val Asn Ile Gln Ala Lys Thr Thr Gly Tyr Asp Thr
35 40

<210> SEQ ID NO 140
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: BLR1 GPCR TM1

<400> SEQUENCE: 140

Met Ala Ser Phe Lys Ala Val Phe Val Pro Val Ala Tyr Ser Leu Ile
1 5 10 15

Phe Leu Leu Gly Val Ile Gly Asn Val Leu Val Leu Val Ile Leu Glu
20 25 30

Arg His Arg Gln Thr Arg Ser Ser Thr Glu
35 40

<210> SEQ ID NO 141
<211> LENGTH: 48
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CL5 AND LCR1 GPCR TM1

<400> SEQUENCE: 141

Arg Glu Glu Asn Ala Asn Phe Asn Lys Ile Phe Leu Pro Thr Ile Tyr
1 5 10 15

Ser Ile Ile Phe Leu Thr Gly Ile Val Gly Asn Gly Leu Val Ile Leu
20 25 30

Val Met Gly Tyr Gln Lys Lys Leu Arg Ser Met Thr Asp Lys Tyr Arg
35 40 45

<210> SEQ ID NO 142
<211> LENGTH: 47
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: EBI1 GPCR TM1

-continued

<400> SEQUENCE: 142

Lys Lys Asp Val Arg Asn Phe Lys Ala Trp Phe Leu Pro Ile Met Tyr
1 5 10 15

Ser Ile Ile Cys Phe Val Gly Leu Leu Gly Asn Gly Leu Val Val Leu
20 25 30

Thr Tyr Ile Tyr Phe Lys Arg Leu Lys Thr Met Thr Asp Thr Tyr
35 40 45

<210> SEQ ID NO 143
<211> LENGTH: 47
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: RBS1rat GPCR TM1

<400> SEQUENCE: 143

Leu Gly Asp Ile Val Ala Phe Gly Thr Ile Phe Leu Ser Ile Phe Tyr
1 5 10 15

Ser Leu Val Phe Thr Phe Gly Leu Val Gly Asn Leu Leu Val Val Leu
20 25 30

Ala Leu Thr Asn Ser Arg Lys Ser Lys Ser Ile Thr Asp Ile Tyr
35 40 45

<210> SEQ ID NO 144
<211> LENGTH: 47
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: EBI2 GPCR TM1

<400> SEQUENCE: 144

Leu Tyr Ala His His Ser Thr Ala Arg Ile Val Met Pro Leu His Tyr
1 5 10 15

Ser Leu Val Phe Ile Ile Gly Leu Val Gly Asn Leu Leu Ala Leu Val
20 25 30

Val Ile Val Gln Asn Arg Lys Lys Ile Asn Ser Thr Thr Leu Tyr
35 40 45

<210> SEQ ID NO 145
<211> LENGTH: 47
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: GCRTchick GPCR TM1

<400> SEQUENCE: 145

Cys Ser Thr Glu Asp Ser Phe Lys Tyr Thr Leu Tyr Gly Cys Val Phe
1 5 10 15

Ser Met Val Phe Val Leu Gly Leu Ile Ala Asn Cys Val Ala Ile Tyr
20 25 30

Ile Phe Thr Phe Thr Leu Lys Val Arg Asn Glu Thr Thr Thr Tyr
35 40 45

<210> SEQ ID NO 146
<211> LENGTH: 46
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: APJ GPCR TM1

-continued

<400> SEQUENCE: 146

Glu Tyr Thr Asp Trp Lys Ser Ser Gly Ala Leu Ile Pro Ala Ile Tyr
1 5 10 15

Met Leu Val Phe Leu Leu Gly Thr Thr Gly Asn Gly Leu Val Leu Trp
20 25 30

Thr Val Phe Arg Ser Ser Arg Glu Lys Arg Arg Ser Ala Asp
35 40 45

<210> SEQ ID NO 147
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: RTArat GPCR TM1

<400> SEQUENCE: 147

Glu Gln Ile Ala Thr Leu Pro Pro Pro Ala Val Thr Asn Tyr Ile Phe
1 5 10 15

Leu Leu Leu Cys Leu Cys Gly Leu Val Gly Asn Gly Leu Val Leu Trp
20 25 30

Phe Phe Gly Phe Ser Ile Lys Arg Thr Pro
35 40

<210> SEQ ID NO 148
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: UHRrat GPCR TM1

<400> SEQUENCE: 148

Ser Leu Gln Leu Val His Gln Leu Lys Gly Leu Ile Val Met Leu Tyr
1 5 10 15

Ser Ile Val Val Val Val Gly Leu Val Gly Asn Cys Leu Leu Val Leu
20 25 30

Val Ile Ala Arg Val Arg Arg Leu His Asn Val
35 40

<210> SEQ ID NO 149
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: FMRL1N GPCR TM1

<400> SEQUENCE: 149

Glu Pro Ala Gly His Thr Val Leu Trp Ile Phe Ser Leu Leu Val His
1 5 10 15

Gly Val Thr Phe Val Phe Gly Val Leu Gly Asn Gly Leu Val Ile Trp
20 25 30

Val Ala Gly Phe Arg Met Thr Arg Thr Val
35 40

<210> SEQ ID NO 150
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: FMRL2 GPCR TM1

-continued

<400> SEQUENCE: 150

Glu Ser Ala Gly Tyr Thr Val Leu Arg Ile Leu Pro Leu Val Val Leu
1 5 10 15

Gly Val Thr Phe Val Leu Gly Val Leu Gly Asn Gly Leu Val Ile Trp
20 25 30

Val Ala Gly Phe Arg Met Thr Arg Thr Val
35 40

<210> SEQ ID NO 151
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: fMLP GPCR TM1

<400> SEQUENCE: 151

Val Ser Ala Gly Tyr Leu Phe Leu Asp Ile Ile Thr Tyr Leu Val Phe
1 5 10 15

Ala Val Thr Phe Val Leu Gly Val Leu Gly Asn Gly Leu Val Ile Trp
20 25 30

Val Ala Gly Phe Arg Met Thr His Thr Val
35 40

<210> SEQ ID NO 152
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLF1catfish GPCR TM1

<400> SEQUENCE: 152

Asn Gly Phe Tyr Asn Ile Pro His Thr Lys Tyr Tyr Tyr Ala Phe Leu
1 5 10 15

Cys Ile Ala Tyr Ala Val Thr Val Leu Gly Asn Ser Phe Ile Met Cys
20 25 30

Thr Ile Tyr Leu Ala Arg Ser Leu His Thr Ala
35 40

<210> SEQ ID NO 153
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLF3catfish GPCR TM1

<400> SEQUENCE: 153

Thr Gly Leu Tyr Asn Ile Pro His Ala Lys Tyr Tyr Tyr Leu Phe Leu
1 5 10 15

Cys Phe Val Tyr Thr Val Thr Phe Leu Gly Asn Ser Phe Ile Met Gly
20 25 30

Thr Ile Tyr Leu Ala Arg Ser Leu His Thr Ala
35 40

<210> SEQ ID NO 154
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLF8catfish GPCR TM1

-continued

<400> SEQUENCE: 154

Gly Phe His Asp Leu Gly Glu Trp Gly Pro Ile Leu Ser Ile Pro Tyr
 1   5   10   15

Leu Leu Met Phe Leu Leu Ser Ser Thr Ser Asn Leu Thr Leu Ile Tyr
            20   25   30

Leu Ile Ile Ser Gln Arg Ala Leu His Ser Pro
        35   40

<210> SEQ ID NO 155
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLF32Acatfish GPCR TM1

<400> SEQUENCE: 155

Ser Gly Phe Ser Gly Ile Pro Phe Ser Gln Tyr Tyr Phe Ala Phe Leu
 1   5   10   15

Ile Phe Ile Tyr Ile Ile Ser Leu Cys Gly Asn Ser Ile Val Leu Phe
            20   25   30

Met Ile Leu Val Asp Arg Thr Leu His Ile Pro
        35   40

<210> SEQ ID NO 156
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLF32Bcatfish, OLF32Ccatfish AND OLF32Dcatfish
      GPCR TM1

<400> SEQUENCE: 156

Ser Gly Phe Ser Gly Ile Pro Phe Ser Gln Tyr Tyr Phe Val Phe Leu
 1   5   10   15

Ile Phe Ile Tyr Ile Ile Ser Leu Cys Gly Asn Ser Ile Val Leu Phe
            20   25   30

Met Ile Leu Val Asp Arg Thr Leu His Ile Pro
        35   40

<210> SEQ ID NO 157
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLF47catfish GPCR TM1

<400> SEQUENCE: 157

Ile Ala Tyr Asn Ser Leu Gly Asn Lys Asn Tyr Leu Ile Leu Ala Leu
 1   5   10   15

Gly Ile Ile Tyr Leu Ile Thr Leu Leu Cys Asn Phe Thr Leu Leu Ala
            20   25   30

Ile Ile Leu Met Asn Ser Ser Leu Gln Asn Pro
        35   40

<210> SEQ ID NO 158
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLF202catfish GPCR TM1

-continued

<400> SEQUENCE: 158

Phe Pro Gly Leu Pro Pro Asn Tyr Tyr Gly Leu Val Ser Val Val Met
1 5 10 15

Phe Phe Val Tyr Val Cys Thr Leu Ile Gly Asn Cys Thr Phe Phe Thr
20 25 30

Leu Phe Leu Arg Glu Lys Ser Leu Gln Lys Pro
35 40

<210> SEQ ID NO 159
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFCOR1chicken GPCR TM1

<400> SEQUENCE: 159

Leu Thr Asp Asn Pro Gly Leu Gln Met Pro Leu Phe Met Val Phe Leu
1 5 10 15

Ala Ile Tyr Thr Ile Thr Leu Leu Thr Asn Leu Gly Leu Ile Ala Leu
20 25 30

Ile Ser Val Asp Leu His Leu Gln Thr Pro
35 40

<210> SEQ ID NO 160
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFCOR2chicken GPCR TM1

<400> SEQUENCE: 160

Leu Thr Asp Asn Pro Arg Leu Gln Met Pro Leu Phe Met Val Phe Leu
1 5 10 15

Val Ile Tyr Thr Thr Thr Leu Leu Thr Asn Leu Gly Leu Ile Ala Leu
20 25 30

Ile Gly Met Asp Leu His Leu Gln Thr Pro
35 40

<210> SEQ ID NO 161
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFCOR3chicken AND OLFCOR4chicken GPCR TM1

<400> SEQUENCE: 161

Leu Thr Asp Asn Pro Gly Leu Gln Met Pro Leu Phe Met Val Phe Leu
1 5 10 15

Ala Ile Tyr Thr Ile Thr Leu Leu Thr Asn Leu Gly Leu Ile Arg Leu
20 25 30

Ile Ser Val Asp Leu His Leu Gln Thr Pro
35 40

<210> SEQ ID NO 162
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFCOR5chicken GPCR TM1

-continued

<400> SEQUENCE: 162

Leu Thr Asp Asn Pro Arg Leu Gln Met Pro Leu Phe Met Val Phe Leu
1 5 10 15

Ala Ile Tyr Thr Ile Thr Leu Leu Ala Asn Leu Gly Leu Ile Ala Leu
20 25 30

Ile Ser Val Asp Phe His Leu Gln Thr Pro
35 40

<210> SEQ ID NO 163
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFCOR6chicken GPCR TM1

<400> SEQUENCE: 163

Leu Thr Asp Asn Pro Gly Leu Gln Met Pro Leu Phe Met Val Phe Leu
1 5 10 15

Ala Ile Tyr Thr Ile Thr Leu Leu Thr Asn Leu Gly Leu Ile Ala Leu
20 25 30

Ile Arg Ile Asp Leu Gln Leu Gln Thr Pro
35 40

<210> SEQ ID NO 164
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFdog GPCR TM1

<400> SEQUENCE: 164

Leu Pro Ile Asp Pro Asp Gln Arg Asp Leu Phe Tyr Ala Leu Phe Leu
1 5 10 15

Ala Met Tyr Val Thr Thr Ile Leu Gly Asn Leu Leu Ile Ile Val Leu
20 25 30

Ile Gln Leu Asp Ser His Leu His Thr Pro
35 40

<210> SEQ ID NO 165
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLF07E GPCR TM1

<400> SEQUENCE: 165

Met Ser Glu Ser Pro Glu Gln Gln Gln Ile Leu Phe Trp Met Phe Leu
1 5 10 15

Ser Met Tyr Leu Val Thr Val Val Gly Asn Val Leu Ile Ile Leu Ala
20 25 30

Ile Ser Ser Asp Ser Arg Leu His Thr Pro
35 40

<210> SEQ ID NO 166
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLF07I GPCR TM1

-continued

<400> SEQUENCE: 166

Leu Pro Ile Gln Pro Glu Gln Gln Asn Leu Cys Tyr Ala Leu Phe Leu
1 5 10 15

Ala Met Tyr Leu Thr Thr Leu Leu Gly Asn Leu Leu Ile Ile Val Leu
20 25 30

Ile Arg Leu Asp Ser His Leu His Thr Pro
35 40

<210> SEQ ID NO 167
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLF07J GPCR TM1

<400> SEQUENCE: 167

Phe Ser Ser Phe His Glu Gln Gln Ile Thr Leu Phe Gly Val Phe Leu
1 5 10 15

Ala Leu Tyr Ile Leu Thr Leu Ala Gly Asn Ile Ile Ile Val Thr Ile
20 25 30

Ile Arg Ile Asp Leu His Leu His Thr Pro
35 40

<210> SEQ ID NO 168
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFOR3mouse GPCR TM1

<400> SEQUENCE: 168

Val Ser Asp His Pro His Leu Glu Ile Ile Phe Phe Ala Val Ile Leu
1 5 10 15

Ala Ser Tyr Leu Leu Thr Leu Val Gly Asn Leu Thr Ile Ile Leu Leu
20 25 30

Ser Arg Leu Asp Ala Arg Leu His Thr Pro
35 40

<210> SEQ ID NO 169
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFrat GPCR TM1

<400> SEQUENCE: 169

Leu Thr Lys Gln Pro Glu Leu Leu Leu Pro Leu Phe Phe Leu Phe Leu
1 5 10 15

Val Ile Tyr Val Leu Thr Val Val Gly Asn Leu Gly Met Ile Leu Leu
20 25 30

Ile Ile Val Ser Pro Leu Leu His Thr Pro
35 40

<210> SEQ ID NO 170
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFF3rat GPCR TM1

-continued

<400> SEQUENCE: 170

Phe Val Glu Asn Lys Asp Leu Gln Pro Leu Ile Tyr Gly Leu Phe Leu
1 5 10 15

Ser Met Tyr Leu Val Thr Val Ile Gly Asn Ile Ser Ile Ile Val Ala
20 25 30

Ile Ile Ser Asp Pro Cys Leu His Thr Pro
35 40

<210> SEQ ID NO 171
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFF5rat GPCR TM1

<400> SEQUENCE: 171

Leu Ser Arg Gln Pro Gln Gln Gln Gln Leu Leu Phe Leu Leu Phe Leu
1 5 10 15

Ile Met Tyr Leu Ala Thr Val Leu Gly Asn Leu Leu Ile Ile Leu Ala
20 25 30

Ile Gly Thr Asp Ser Arg Leu His Thr Pro
35 40

<210> SEQ ID NO 172
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFF6rat GPCR TM1

<400> SEQUENCE: 172

Phe Pro Gly Pro Arg Ser Met Arg Ile Gly Leu Phe Leu Leu Phe Leu
1 5 10 15

Val Met Tyr Leu Leu Thr Val Val Gly Asn Leu Ala Ile Ile Ser Leu
20 25 30

Val Gly Ala His Arg Cys Leu Gln Thr Pro
35 40

<210> SEQ ID NO 173
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFF12rat GPCR TM1

<400> SEQUENCE: 173

Phe Thr Glu Asn Pro Gln Leu His Phe Leu Ile Phe Ala Leu Phe Leu
1 5 10 15

Ser Met Tyr Leu Val Thr Val Leu Gly Asn Leu Leu Ile Ile Met Ala
20 25 30

Ile Ile Thr Gln Ser His Leu His Thr Pro
35 40

<210> SEQ ID NO 174
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFI3rat GPCR TM1

-continued

<400> SEQUENCE: 174

Leu Pro Ile Pro Glu Glu His Gln His Leu Phe Tyr Ala Leu Phe Leu
1 5 10 15

Val Met Tyr Leu Thr Thr Ile Leu Gly Asn Leu Leu Ile Ile Val Leu
20 25 30

Val Gln Leu Asp Ser Gln Leu His Thr Pro
35 40

<210> SEQ ID NO 175
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFI7rat GPCR TM1
<221> NAME/KEY: MOD_RES
<222> LOCATION: (18)...(18)
<223> OTHER INFORMATION: Xaa = unknown amino acid

<400> SEQUENCE: 175

Phe Pro Ala Pro Ala Pro Leu Arg Val Leu Leu Phe Phe Leu Ser Leu
1 5 10 15

Leu Xaa Tyr Val Leu Val Leu Thr Glu Asn Met Leu Ile Ile Ile Ala
20 25 30

Ile Arg Asn His Pro Thr Leu His Lys Pro
35 40

<210> SEQ ID NO 176
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFI8rat GPCR TM1

<400> SEQUENCE: 176

Leu Pro Ile Pro Pro Glu His Gln Gln Leu Phe Phe Ala Leu Phe Leu
1 5 10 15

Ile Met Tyr Leu Thr Thr Phe Leu Gly Asn Leu Leu Ile Val Val Leu
20 25 30

Val Gln Leu Asp Ser His Leu His Thr Pro
35 40

<210> SEQ ID NO 177
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFI9rat GPCR TM1

<400> SEQUENCE: 177

Leu Pro Phe Pro Pro Glu Tyr Gln His Leu Phe Tyr Ala Leu Phe Leu
1 5 10 15

Ala Met Tyr Leu Thr Thr Leu Leu Gly Asn Leu Ile Ile Ile Ile Leu
20 25 30

Ile Leu Leu Asp Ser His Leu His Thr Pro
35 40

<210> SEQ ID NO 178
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFI14rat GPCR TM1

-continued

<400> SEQUENCE: 178

Leu Pro Ile Pro Ser Glu Tyr His Leu Leu Phe Tyr Ala Leu Phe Leu
1 5 10 15

Ala Met Tyr Leu Thr Ile Ile Leu Gly Asn Leu Leu Ile Ile Val Leu
20 25 30

Val Arg Leu Asp Ser His Leu His Met Pro
35 40

<210> SEQ ID NO 179
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFI15rat GPCR TM1

<400> SEQUENCE: 179

Leu Pro Ile Pro Ser Glu His Gln His Val Phe Tyr Ala Leu Phe Leu
1 5 10 15

Ser Met Tyr Leu Thr Thr Val Leu Gly Asn Leu Ile Ile Ile Ile Leu
20 25 30

Ile His Leu Asp Ser His Leu His Thr Pro
35 40

<210> SEQ ID NO 180
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFOR17_40 GPCR TM1

<400> SEQUENCE: 180

Leu Leu Glu Ala Pro Gly Leu Gln Pro Val Val Phe Val Leu Phe Leu
1 5 10 15

Phe Ala Tyr Leu Val Thr Val Arg Gly Asn Leu Ser Ile Leu Ala Ala
20 25 30

Val Leu Val Glu Pro Lys Leu His Thr Pro
35 40

<210> SEQ ID NO 181
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: GUST27rat GPCR TM1

<400> SEQUENCE: 181

Met Ile Leu Asn Cys Asn Pro Phe Ser Gly Leu Phe Leu Ser Met Tyr
1 5 10 15

Leu Val Thr Val Leu Gly Asn Leu Leu Ile Ile Leu Ala Val Ser Ser
20 25 30

Asn Ser His Leu His Asn Leu
35

<210> SEQ ID NO 182
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: RPE GPCR TM1

-continued

<400> SEQUENCE: 182

Pro Thr Gly Phe Gly Glu Leu Glu Val Leu Ala Val Gly Met Val Leu
1 5 10 15

Leu Val Glu Ala Leu Ser Gly Leu Ser Leu Asn Thr Leu Thr Ile Phe
20 25 30

Ser Phe Cys Lys Thr Pro Glu Leu Arg Thr Pro
35 40

<210> SEQ ID NO 183
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: HHRF1 GPCR TM1

<400> SEQUENCE: 183

Phe Thr Asp Val Leu Asn Gln Ser Lys Pro Val Thr Leu Phe Leu Tyr
1 5 10 15

Gly Val Val Phe Leu Phe Gly Ser Ile Gly Asn Phe Leu Val Ile Phe
20 25 30

Thr Ile Thr Trp Arg Arg Arg Ile Gln Cys Ser
35 40

<210> SEQ ID NO 184
<211> LENGTH: 44
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: HHRF2 GPCR TM1

<400> SEQUENCE: 184

Asn Ser Thr Glu Ile Tyr Gln Leu Phe Glu Tyr Thr Arg Leu Gly Val
1 5 10 15

Trp Leu Met Cys Ile Val Gly Thr Phe Leu Asn Val Leu Val Ile Thr
20 25 30

Thr Ile Leu Tyr Tyr Arg Arg Lys Lys Lys Ser Pro
35 40

<210> SEQ ID NO 185
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: HHRF3 GPCR TM1

<400> SEQUENCE: 185

Met Thr Gly Pro Leu Phe Ala Ile Arg Thr Thr Glu Ala Val Leu Asn
1 5 10 15

Thr Phe Ile Ile Phe Val Gly Gly Pro Leu Asn Ala Ile Val Leu Ile
20 25 30

Thr Gln Leu Leu Thr Asn Arg Val Leu Gly Tyr Ser Thr
35 40 45

<210> SEQ ID NO 186
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: MCP-1A AND MCP-1B GPCR TM1

-continued

<400> SEQUENCE: 186

Asp Val Lys Gln Ile Gly Ala Gln Leu Leu Pro Pro Leu Tyr Ser Leu
1 5 10 15

Val Phe Ile Phe Gly Phe Val Gly Asn Met Leu Val Val Leu Ile Leu
20 25 30

Ile Asn Cys Lys Lys Leu Lys Cys Leu
35 40

<210> SEQ ID NO 187
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PPR1bovine GPCR TM1

<400> SEQUENCE: 187

Glu Val Arg Lys Phe Ala Lys Val Phe Leu Pro Ala Phe Phe Thr Ile
1 5 10 15

Ala Phe Ile Ile Gly Leu Ala Gly Asn Ser Thr Val Val Ala Ile Tyr
20 25 30

Ala Tyr Tyr Lys Lys Arg Arg Thr Lys
35 40

<210> SEQ ID NO 188
<211> LENGTH: 50
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: GPCRAelegans GPCR TM2

<400> SEQUENCE: 188

Thr Arg Phe Leu Met Cys Asn Leu Ala Phe Ala Asp Phe Ile Leu Gly
1 5 10 15

Leu Tyr Ile Phe Ile Leu Thr Ser Val Ser Ala Val Thr Arg Gly Asp
20 25 30

Tyr His Asn Tyr Val Gln Gln Trp Gln Asn Gly Ala Gly Cys Lys Ile
35 40 45

Leu Gly
50

<210> SEQ ID NO 189
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: GRH GPCR TM2

<400> SEQUENCE: 189

Lys Leu Leu Leu Lys His Leu Thr Leu Ala Asn Leu Leu Glu Thr Leu
1 5 10 15

Ile Val Met Pro Leu Asp Gly Met Trp Asn Ile Thr Val Gln Trp Tyr
20 25 30

Ala Gly Glu Leu Leu Cys Lys Val Leu Ser
35 40

<210> SEQ ID NO 190
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: TRH GPCR TM2

-continued

<400> SEQUENCE: 190

Thr Asn Cys Tyr Leu Val Ser Leu Ala Val Ala Asp Leu Met Val Leu
1 5 10 15

Val Ala Ala Gly Leu Pro Asn Ile Thr Asp Ser Ile Tyr Gly Ser Trp
20 25 30

Val Tyr Gly Tyr Val Gly Cys Leu Cys Ile Thr
35 40

<210> SEQ ID NO 191
<211> LENGTH: 50
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: FSHprec GPCR TM2

<400> SEQUENCE: 191

Pro Arg Phe Leu Met Cys Asn Leu Ala Phe Ala Asp Leu Cys Ile Gly
1 5 10 15

Ile Tyr Leu Leu Leu Ile Ala Ser Val Asp Ile His Thr Lys Ser Gln
20 25 30

Tyr His Asn Tyr Ala Ile Asp Trp Gln Thr Gly Ala Gly Cys Asp Ala
35 40 45

Ala Gly
50

<210> SEQ ID NO 192
<211> LENGTH: 50
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: TSHprec GPCR TM2

<400> SEQUENCE: 192

Pro Arg Phe Leu Met Cys Asn Leu Ala Phe Ala Asp Phe Cys Met Gly
1 5 10 15

Met Tyr Leu Leu Leu Ile Ala Ser Val Asp Leu Tyr Thr His Ser Glu
20 25 30

Tyr Tyr Asn His Ala Ile Asp Trp Gln Thr Gly Pro Gly Cys Asn Thr
35 40 45

Ala Gly
50

<210> SEQ ID NO 193
<211> LENGTH: 50
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: LH_CGprec GPCR TM2

<400> SEQUENCE: 193

Pro Arg Phe Leu Met Cys Asn Leu Ser Phe Ala Asp Phe Cys Met Gly
1 5 10 15

Leu Tyr Leu Leu Leu Ile Ala Ser Val Asp Ser Gln Thr Lys Gly Gln
20 25 30

Tyr Tyr Asn His Ala Ile Asp Trp Gln Thr Gly Ser Gly Cys Ser Thr
35 40 45

Ala Gly
50

-continued

<210> SEQ ID NO 194
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PGE_EP1 GPCR TM2

<400> SEQUENCE: 194

Phe Leu Leu Phe Val Ala Ser Leu Leu Ala Thr Asp Leu Ala Gly His
 1   5   10   15

Val Ile Pro Gly Ala Leu Val Leu Arg Leu Tyr Thr Ala Gly Arg Ala
            20   25   30

Pro Ala Gly Gly Ala Cys His Phe Leu Gly
        35   40

<210> SEQ ID NO 195
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PGE_EP2 GPCR TM2

<400> SEQUENCE: 195

Phe Tyr Thr Leu Val Cys Gly Leu Ala Val Thr Asp Leu Leu Gly Thr
 1   5   10   15

Leu Leu Val Ser Pro Val Thr Ile Ala Thr Tyr Met Lys Gly Gln Trp
            20   25   30

Pro Gly Gly Gln Pro Leu Cys Glu Tyr Ser Thr
        35   40

<210> SEQ ID NO 196
<211> LENGTH: 47
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PGE_EP3 GPCR TM2

<400> SEQUENCE: 196

Phe His Val Leu Val Thr Glu Leu Val Phe Thr Asp Leu Leu Gly Thr
 1   5   10   15

Cys Leu Ile Ser Pro Val Val Leu Ala Ser Tyr Ala Arg Asn Gln Thr
            20   25   30

Leu Val Ala Leu Ala Pro Glu Ser Arg Ala Cys Thr Tyr Phe Ala
        35   40   45

<210> SEQ ID NO 197
<211> LENGTH: 47
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PGF GPCR TM2

<400> SEQUENCE: 197

Phe Leu Leu Leu Ala Ser Gly Leu Val Ile Thr Asp Phe Phe Gly His
 1   5   10   15

Leu Ile Asn Gly Ala Ile Ala Val Phe Val Tyr Ala Ser Asp Lys Glu
            20   25   30

Trp Ile Arg Phe Asp Gln Ser Asn Val Leu Cys Ser Ile Phe Gly
        35   40   45

-continued

<210> SEQ ID NO 198
<211> LENGTH: 48
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PGI GPCR TM2

<400> SEQUENCE: 198

Phe Ala Val Leu Val Thr Gly Leu Ala Ala Thr Asp Leu Leu Gly Thr
1 5 10 15

Ser Phe Leu Ser Pro Ala Val Phe Val Ala Tyr Ala Arg Asn Ser Ser
20 25 30

Leu Leu Gly Leu Ala Arg Gly Gly Pro Ala Leu Cys Asp Ala Phe Ala
35 40 45

<210> SEQ ID NO 199
<211> LENGTH: 47
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: TXA2 GPCR TM2

<400> SEQUENCE: 199

Phe Leu Thr Phe Leu Cys Gly Leu Val Leu Thr Asp Phe Leu Gly Leu
1 5 10 15

Leu Val Thr Gly Thr Ile Val Val Ser Gln His Ala Ala Leu Phe Glu
20 25 30

Trp His Ala Val Asp Pro Gly Cys Arg Leu Cys Arg Phe Met Gly
35 40 45

<210> SEQ ID NO 200
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PAF GPCR TM2

<400> SEQUENCE: 200

Ile Phe Met Val Asn Leu Thr Met Ala Asp Met Leu Phe Leu Ile Thr
1 5 10 15

Leu Pro Leu Trp Ile Val Tyr Tyr Gln Asn Gln Gly Asn Trp Ile Leu
20 25 30

Pro Lys Phe Leu Cys Asn Val Ala Gly
35 40

<210> SEQ ID NO 201
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: M2 GPCR TM2

<400> SEQUENCE: 201

Asn Asn Tyr Phe Leu Phe Ser Leu Ala Cys Ala Asp Leu Ile Ile Gly
1 5 10 15

Val Phe Ser Met Asn Leu Tyr Thr Leu Tyr Thr Val Ile Gly Tyr Trp
20 25 30

Pro Leu Gly Pro Val Val Cys Asp Leu Trp Leu
35 40

-continued

<210> SEQ ID NO 202
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: M4 GPCR TM2

<400> SEQUENCE: 202

Asn Asn Tyr Phe Leu Phe Ser Leu Ala Cys Ala Asp Leu Ile Ile Gly
1 5 10 15

Ala Phe Ser Met Asn Leu Tyr Thr Val Tyr Ile Ile Lys Gly Tyr Trp
20 25 30

Pro Leu Gly Ala Val Val Cys Asp Leu Trp Leu
35 40

<210> SEQ ID NO 203
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: M1 GPCR TM2

<400> SEQUENCE: 203

Asn Asn Tyr Phe Leu Leu Ser Leu Ala Cys Ala Asp Leu Ile Ile Gly
1 5 10 15

Thr Phe Ser Met Asn Leu Tyr Thr Thr Tyr Leu Leu Met Gly His Trp
20 25 30

Ala Leu Gly Thr Leu Ala Cys Asp Leu Trp Leu
35 40

<210> SEQ ID NO 204
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: M3 GPCR TM2

<400> SEQUENCE: 204

Asn Asn Tyr Phe Leu Leu Ser Leu Ala Cys Ala Asp Leu Ile Ile Gly
1 5 10 15

Val Ile Ser Met Asn Leu Phe Thr Thr Tyr Ile Ile Met Asn Arg Trp
20 25 30

Ala Leu Gly Asn Leu Ala Cys Asp Leu Trp Leu
35 40

<210> SEQ ID NO 205
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: M5 GPCR TM2

<400> SEQUENCE: 205

Asn Asn Tyr Tyr Leu Leu Ser Leu Ala Cys Ala Asp Leu Ile Ile Gly
1 5 10 15

Ile Phe Ser Met Asn Leu Tyr Thr Thr Tyr Ile Leu Met Gly Arg Trp
20 25 30

Ala Leu Gly Ser Leu Ala Cys Asp Leu Trp Leu
35 40

-continued

<210> SEQ ID NO 206
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: H1 GPCR TM2

<400> SEQUENCE: 206

Gly Asn Leu Tyr Ile Val Ser Leu Ser Val Ala Asp Leu Ile Val Gly
1 5 10 15

Ala Val Val Met Pro Met Asn Ile Leu Tyr Leu Leu Met Ser Lys Trp
20 25 30

Ser Leu Gly Arg Pro Leu Cys Leu Phe Trp Leu
35 40

<210> SEQ ID NO 207
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: H2 GPCR TM2

<400> SEQUENCE: 207

Thr Asn Cys Phe Ile Val Ser Leu Ala Ile Thr Asp Leu Leu Leu Gly
1 5 10 15

Leu Leu Val Leu Pro Phe Ser Ala Ile Tyr Gln Leu Ser Cys Lys Trp
20 25 30

Ser Phe Gly Lys Val Phe Cys Asn Ile Tyr Thr
35 40

<210> SEQ ID NO 208
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT1A GPCR TM2

<400> SEQUENCE: 208

Ala Asn Tyr Leu Ile Gly Ser Leu Ala Val Thr Asp Leu Met Val Ser
1 5 10 15

Val Leu Val Leu Pro Met Ala Ala Leu Tyr Gln Val Leu Asn Lys Trp
20 25 30

Thr Leu Gly Gln Val Thr Cys Asp Leu Phe Ile
35 40

<210> SEQ ID NO 209
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT1B GPCR TM2

<400> SEQUENCE: 209

Ala Asn Tyr Leu Ile Ala Ser Leu Ala Val Thr Asp Leu Leu Val Ser
1 5 10 15

Ile Leu Val Met Pro Ile Ser Thr Met Tyr Thr Val Thr Gly Arg Trp
20 25 30

Thr Leu Gly Gln Val Val Cys Asp Phe Trp Leu
35 40

-continued

<210> SEQ ID NO 210
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT1D GPCR TM2

<400> SEQUENCE: 210

Ala Asn Tyr Leu Ile Gly Ser Leu Ala Thr Thr Asp Leu Leu Val Ser
1 5 10 15

Ile Leu Val Met Pro Ile Ser Ile Ala Tyr Thr Ile Thr His Thr Trp
20 25 30

Asn Phe Gly Gln Ile Leu Cys Asp Ile Trp Leu
35 40

<210> SEQ ID NO 211
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT1E GPCR TM2

<400> SEQUENCE: 211

Ala Asn Tyr Leu Ile Cys Ser Leu Ala Val Thr Asp Leu Leu Val Ala
1 5 10 15

Val Leu Val Met Pro Leu Ser Ile Ile Tyr Ile Val Met Asp Arg Trp
20 25 30

Lys Leu Gly Tyr Phe Leu Cys Glu Val Trp Leu
35 40

<210> SEQ ID NO 212
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT1F GPCR TM2

<400> SEQUENCE: 212

Ala Asn Tyr Leu Ile Cys Ser Leu Ala Val Thr Asp Phe Leu Val Ala
1 5 10 15

Val Leu Val Met Pro Phe Ser Ile Val Tyr Ile Val Arg Glu Ser Trp
20 25 30

Ile Met Gly Gln Val Val Cys Asp Ile Trp Leu
35 40

<210> SEQ ID NO 213
<211> LENGTH: 44
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT2A GPCR TM2

<400> SEQUENCE: 213

Thr Asn Tyr Phe Leu Met Ser Leu Ala Ile Ala Asp Met Leu Leu Gly
1 5 10 15

Phe Leu Val Met Pro Val Ser Met Leu Thr Ile Leu Tyr Gly Tyr Arg
20 25 30

Trp Pro Leu Pro Ser Lys Leu Cys Ala Val Trp Ile
35 40

-continued

<210> SEQ ID NO 214
<211> LENGTH: 44
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT2B GPCR TM2

<400> SEQUENCE: 214

Thr Asn Tyr Phe Leu Met Ser Leu Ala Val Ala Asp Leu Leu Val Gly
 1   5   10   15

Leu Phe Val Met Pro Ile Ala Leu Leu Thr Ile Met Phe Glu Ala Met
            20   25   30

Trp Pro Leu Pro Leu Val Leu Cys Pro Ala Trp Leu
        35   40

<210> SEQ ID NO 215
<211> LENGTH: 44
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT2C GPCR TM2

<400> SEQUENCE: 215

Thr Asn Tyr Phe Leu Met Ser Leu Ala Ile Ala Asp Met Leu Val Gly
 1   5   10   15

Leu Leu Val Met Pro Leu Ser Leu Leu Ala Ile Leu Tyr Asp Tyr Val
            20   25   30

Trp Pro Leu Pro Arg Tyr Leu Cys Pro Val Trp Ile
        35   40

<210> SEQ ID NO 216
<211> LENGTH: 44
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT5A GPCR TM2

<400> SEQUENCE: 216

Pro His Asn Leu Val Ala Ser Met Ala Val Ser Asp Val Leu Val Ala
 1   5   10   15

Ala Leu Val Met Pro Leu Ser Leu Val His Glu Leu Ser Gly Arg Arg
            20   25   30

Trp Gln Leu Gly Arg Arg Leu Cys Gln Leu Trp Ile
        35   40

<210> SEQ ID NO 217
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT5Brat GPCR TM2

<400> SEQUENCE: 217

Pro His Asn Leu Val Ala Ser Thr Ala Val Ser Asp Val Leu Val Ala
 1   5   10   15

Ala Leu Val Met Pro Leu Ser Leu Val Ser Glu Leu Ser Ala Gly Arg
            20   25   30

Arg Trp Gln Leu Gly Arg Ser Leu Cys His Val Trp Ile
        35   40   45

-continued

<210> SEQ ID NO 218
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT6rat GPCR TM2

<400> SEQUENCE: 218

Ser Asn Phe Phe Leu Val Ser Leu Phe Thr Ser Asp Leu Met Val Gly
 1   5   10   15

Leu Val Val Met Pro Pro Ala Met Leu Asn Ala Leu Tyr Gly Arg Trp
            20   25   30

Val Leu Ala Arg Gly Leu Cys Leu Leu Trp Thr
        35   40

<210> SEQ ID NO 219
<211> LENGTH: 44
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 5HT7 GPCR TM2

<400> SEQUENCE: 219

Ser Asn Tyr Leu Ile Val Ser Leu Ala Leu Ala Asp Leu Ser Val Ala
 1   5   10   15

Val Ala Val Met Pro Phe Val Ser Val Thr Asp Leu Ile Gly Gly Lys
            20   25   30

Trp Ile Phe Gly His Phe Phe Cys Asn Val Phe Ile
        35   40

<210> SEQ ID NO 220
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: alpha1A GPCR TM2

<400> SEQUENCE: 220

Thr Asn Tyr Phe Ile Val Asn Leu Ala Val Ala Asp Leu Leu Leu Ser
 1   5   10   15

Ala Thr Val Leu Pro Phe Ser Ala Thr Met Glu Val Leu Gly Phe Trp
            20   25   30

Ala Phe Gly Arg Ala Phe Cys Asp Val Trp Ala
        35   40

<210> SEQ ID NO 221
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: alpha1B GPCR TM2

<400> SEQUENCE: 221

Thr Asn Tyr Phe Ile Val Asn Leu Ala Met Ala Asp Leu Leu Leu Ser
 1   5   10   15

Phe Thr Val Leu Pro Phe Ser Ala Ala Leu Glu Val Leu Gly Tyr Trp
            20   25   30

Val Leu Gly Arg Ile Phe Cys Asp Ile Trp Ala
        35   40

-continued

<210> SEQ ID NO 222
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: alpha1C GPCR TM2

<400> SEQUENCE: 222

Thr His Tyr Tyr Ile Val Asn Leu Ala Val Ala Asp Leu Leu Leu Thr
1 5 10 15

Ser Thr Val Leu Pro Phe Ser Ala Ile Phe Glu Val Leu Gly Tyr Trp
20 25 30

Ala Phe Gly Arg Val Phe Cys Asn Ile Trp Ala
35 40

<210> SEQ ID NO 223
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: alpha2A GPCR TM2

<400> SEQUENCE: 223

Gln Asn Leu Phe Leu Val Ser Leu Ala Ser Ala Asp Ile Leu Val Ala
1 5 10 15

Thr Leu Val Ile Pro Phe Ser Leu Ala Asn Glu Val Met Gly Tyr Trp
20 25 30

Tyr Phe Gly Lys Ala Trp Cys Glu Ile Tyr Leu
35 40

<210> SEQ ID NO 224
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: alpha2B GPCR TM2

<400> SEQUENCE: 224

Gln Asn Leu Phe Leu Val Ser Leu Ala Ala Ala Asp Ile Leu Val Ala
1 5 10 15

Thr Leu Ile Ile Pro Phe Ser Leu Ala Asn Glu Leu Leu Gly Tyr Trp
20 25 30

Tyr Phe Arg Arg Thr Trp Cys Glu Val Tyr Leu
35 40

<210> SEQ ID NO 225
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: alpha2C1 AND alpha2C2 GPCR TM2

<400> SEQUENCE: 225

Gln Asn Leu Phe Leu Val Ser Leu Ala Ser Ala Asp Ile Leu Val Ala
1 5 10 15

Thr Leu Val Met Pro Phe Ser Leu Ala Asn Glu Leu Met Ala Tyr Trp
20 25 30

Tyr Phe Gly Gln Val Trp Cys Gly Val Tyr Leu
35 40

-continued

<210> SEQ ID NO 226
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: beta1 GPCR TM2

<400> SEQUENCE: 226

Thr Asn Leu Phe Ile Met Ser Leu Ala Ser Ala Asp Leu Val Met Gly
1 5 10 15

Leu Leu Val Val Pro Phe Gly Ala Thr Ile Val Val Trp Gly Arg Trp
20 25 30

Glu Tyr Gly Ser Phe Phe Cys Glu Leu Trp Thr
35 40

<210> SEQ ID NO 227
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: beta2 GPCR TM2

<400> SEQUENCE: 227

Thr Asn Tyr Phe Ile Thr Ser Leu Ala Cys Ala Asp Leu Val Met Gly
1 5 10 15

Leu Ala Val Val Pro Phe Gly Ala Ala His Ile Leu Met Lys Met Trp
20 25 30

Thr Phe Gly Asn Phe Trp Cys Glu Phe Trp Thr
35 40

<210> SEQ ID NO 228
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: beta3 GPCR TM2

<400> SEQUENCE: 228

Thr Asn Val Phe Val Thr Ser Leu Ala Ala Ala Asp Leu Val Met Gly
1 5 10 15

Leu Leu Val Val Pro Pro Ala Ala Thr Leu Ala Leu Thr Gly His Trp
20 25 30

Pro Leu Gly Ala Thr Gly Cys Glu Leu Trp Thr
35 40

<210> SEQ ID NO 229
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: beta4turkey GPCR TM2

<400> SEQUENCE: 229

Thr Asn Val Phe Val Thr Ser Leu Ala Cys Ala Asp Leu Val Met Gly
1 5 10 15

Leu Leu Val Val Pro Pro Gly Ala Thr Ile Leu Leu Ser Gly His Trp
20 25 30

Pro Tyr Gly Thr Val Val Cys Glu Leu Trp Thr
35 40

-continued

<210> SEQ ID NO 230
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: D1A GPCR TM2

<400> SEQUENCE: 230

Thr Asn Phe Phe Val Ile Ser Leu Ala Val Ser Asp Leu Leu Val Ala
1 5 10 15

Val Leu Val Met Pro Trp Lys Ala Val Ala Glu Ile Ala Gly Phe Trp
20 25 30

Pro Phe Gly Ser Phe Cys Asn Ile Trp Val
35 40

<210> SEQ ID NO 231
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: D2 GPCR TM2

<400> SEQUENCE: 231

Thr Asn Tyr Leu Ile Val Ser Leu Ala Val Ala Asp Leu Leu Val Ala
1 5 10 15

Thr Leu Val Met Pro Trp Val Val Tyr Leu Glu Val Val Gly Glu Trp
20 25 30

Lys Phe Ser Arg Ile His Cys Asp Ile Phe Val
35 40

<210> SEQ ID NO 232
<211> LENGTH: 44
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: D3 GPCR TM2

<400> SEQUENCE: 232

Thr Asn Tyr Leu Val Val Ser Leu Ala Val Ala Asp Leu Leu Val Ala
1 5 10 15

Thr Leu Val Met Pro Trp Val Val Tyr Leu Glu Val Thr Gly Gly Val
20 25 30

Trp Asn Phe Ser Arg Ile Cys Cys Asp Val Phe Val
35 40

<210> SEQ ID NO 233
<211> LENGTH: 44
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: D4 GPCR TM2

<400> SEQUENCE: 233

Thr Asn Ser Phe Ile Val Ser Leu Ala Ala Ala Asp Leu Leu Leu Ala
1 5 10 15

Leu Leu Val Leu Pro Leu Phe Val Tyr Ser Glu Val Gln Gly Gly Ala
20 25 30

Trp Leu Leu Ser Pro Arg Leu Cys Asp Ala Leu Met
35 40

-continued

<210> SEQ ID NO 234
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: D5 GPCR TM2

<400> SEQUENCE: 234

Thr Asn Val Phe Ile Val Ser Leu Ala Val Ser Asp Leu Phe Val Ala
1 5 10 15

Leu Leu Val Met Pro Trp Lys Ala Val Ala Glu Val Ala Gly Tyr Trp
20 25 30

Pro Phe Gly Ala Phe Cys Asp Val Trp Val
35 40

<210> SEQ ID NO 235
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: A1 GPCR TM2

<400> SEQUENCE: 235

Thr Phe Cys Phe Ile Val Ser Leu Ala Val Ala Asp Val Ala Val Gly
1 5 10 15

Ala Leu Val Ile Pro Leu Ala Ile Leu Ile Asn Ile Gly Pro Gln Thr
20 25 30

Tyr Phe His Thr Cys Leu Met Val Ala
35 40

<210> SEQ ID NO 236
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: A2a GPCR TM2

<400> SEQUENCE: 236

Thr Asn Tyr Phe Val Val Ser Leu Ala Ala Ala Asp Ile Ala Val Gly
1 5 10 15

Val Leu Ala Ile Pro Phe Ala Ile Thr Ile Ser Thr Gly Phe Cys Ala
20 25 30

Ala Cys His Gly Cys Leu Phe Ile Ala
35 40

<210> SEQ ID NO 237
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: A2b GPCR TM2

<400> SEQUENCE: 237

Thr Asn Tyr Phe Leu Val Ser Leu Ala Ala Ala Asp Val Ala Val Gly
1 5 10 15

Leu Phe Ala Ile Pro Phe Ala Ile Thr Ile Ser Leu Gly Phe Cys Thr
20 25 30

Asp Phe Tyr Gly Cys Leu Phe Leu Ala
35 40

-continued

<210> SEQ ID NO 238
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: A3 GPCR TM2

<400> SEQUENCE: 238

Thr Phe Tyr Phe Ile Val Ser Leu Ala Leu Ala Asp Ile Ala Val Gly
1 5 10 15

Val Leu Val Met Pro Leu Ala Ile Val Val Ser Leu Gly Ile Thr Ile
20 25 30

His Phe Tyr Ser Cys Leu Phe Met Thr
35 40

<210> SEQ ID NO 239
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OCdrome GPCR TM2

<400> SEQUENCE: 239

Gln Asn Phe Phe Ile Val Ser Leu Ala Val Ala Asp Leu Thr Val Ala
1 5 10 15

Leu Leu Val Leu Pro Phe Asn Val Ala Tyr Ser Ile Leu Gly Arg Trp
20 25 30

Glu Phe Gly Ile His Leu Cys Lys Leu Trp Leu
35 40

<210> SEQ ID NO 240
<211> LENGTH: 49
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: ACTH GPCR TM2

<400> SEQUENCE: 240

Met Tyr Phe Phe Ile Cys Ser Leu Ala Ile Ser Asp Met Leu Gly Ser
1 5 10 15

Leu Tyr Lys Ile Leu Glu Asn Ile Leu Ile Ile Leu Arg Asn Met Gly
20 25 30

Tyr Leu Lys Pro Arg Gly Ser Phe Glu Thr Thr Ala Asp Asp Ile Ile
35 40 45

Asp

<210> SEQ ID NO 241
<211> LENGTH: 49
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: MSH GPCR TM2

<400> SEQUENCE: 241

Met Tyr Cys Phe Ile Cys Cys Leu Ala Leu Ser Asp Leu Leu Val Ser
1 5 10 15

Gly Thr Asn Val Leu Glu Thr Ala Val Ile Leu Leu Leu Glu Ala Gly
20 25 30

Ala Leu Val Ala Arg Ala Ala Val Leu Gln Gln Leu Asp Asn Val Ile
35 40 45

Asp

-continued

<210> SEQ ID NO 242
<211> LENGTH: 49
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: MC3 GPCR TM2

<400> SEQUENCE: 242

Met Tyr Phe Phe Leu Cys Ser Leu Ala Val Ala Asp Met Leu Val Ser
1 5 10 15

Val Ser Asn Ala Leu Glu Thr Ile Met Ile Ala Ile Val His Ser Asp
20 25 30

Asp Tyr Thr Phe Glu Asp Gln Phe Ile Gln His Met Asp Asn Ile Phe
35 40 45

Asp

<210> SEQ ID NO 243
<211> LENGTH: 48
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: MC4 GPCR TM2

<400> SEQUENCE: 243

Met Tyr Phe Phe Ile Cys Ser Leu Ala Val Ala Asp Met Leu Val Ser
1 5 10 15

Val Ser Asn Gly Ser Glu Thr Ile Ile Ile Thr Leu Leu Asn Ser Thr
20 25 30

Asp Thr Asp Ala Gln Ser Phe Thr Val Asn Ile Asp Asn Val Ile Asp
35 40 45

<210> SEQ ID NO 244
<211> LENGTH: 49
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: MC5 GPCR TM2

<400> SEQUENCE: 244

Met Tyr Phe Phe Val Cys Ser Leu Ala Val Ala Asp Met Leu Val Ser
1 5 10 15

Met Ser Ser Ala Trp Glu Thr Ile Thr Ile Tyr Leu Leu Asn Asn Lys
20 25 30

His Leu Val Ile Ala Asp Ala Phe Val Arg His Ile Asp Asn Val Phe
35 40 45

Asp

<210> SEQ ID NO 245
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: melatonin GPCR TM2

<400> SEQUENCE: 245

Gly Asn Ile Phe Val Val Ser Leu Ala Val Ala Asp Leu Val Val Ala
1 5 10 15

Ile Tyr Pro Tyr Pro Leu Val Leu Met Ser Ile Phe Asn Asn Gly Trp
20 25 30

Asn Leu Gly Tyr Leu His Cys Gln Val Ser Gly
35 40

-continued

<210> SEQ ID NO 246
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: oxytocin GPCR TM2

<400> SEQUENCE: 246

Leu Phe Phe Phe Met Lys His Leu Ser Ile Ala Asp Leu Val Val Ala
1 5 10 15

Val Phe Gln Val Leu Pro Gln Leu Leu Trp Asp Ile Thr Phe Arg Phe
20 25 30

Tyr Gly Pro Asp Leu Leu Cys Arg Leu Val Lys
35 40

<210> SEQ ID NO 247
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: conopressinLs GPCR TM2

<400> SEQUENCE: 247

Met Gln Trp Phe Ile Ala His Leu Ala Phe Ala Asp Ile Phe Val Gly
1 5 10 15

Phe Phe Asn Ile Leu Pro Gln Leu Ile Ser Asp Val Thr Ile Val Phe
20 25 30

His Gly Asp Asp Phe Thr Cys Arg Phe Ile Lys
35 40

<210> SEQ ID NO 248
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: V1A GPCR TM2

<400> SEQUENCE: 248

Met His Leu Phe Ile Arg His Leu Ser Leu Ala Asp Leu Ala Val Ala
1 5 10 15

Phe Phe Gln Val Leu Pro Gln Met Cys Trp Asp Ile Thr Tyr Arg Phe
20 25 30

Arg Gly Pro Asp Trp Leu Cys Arg Val Val Lys
35 40

<210> SEQ ID NO 249
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: V1B GPCR TM2

<400> SEQUENCE: 249

Met His Leu Phe Val Leu His Leu Ala Leu Thr Asp Leu Ala Val Ala
1 5 10 15

Leu Phe Gln Val Leu Pro Gln Leu Leu Trp Asp Ile Thr Tyr Arg Phe
20 25 30

Gln Gly Pro Asp Leu Leu Cys Arg Ala Val Lys
35 40

-continued

<210> SEQ ID NO 250
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: V2 GPCR TM2

<400> SEQUENCE: 250

Ile His Val Phe Ile Gly His Leu Cys Leu Ala Asp Leu Ala Val Ala
1 5 10 15

Leu Phe Gln Val Leu Pro Gln Leu Ala Trp Lys Ala Thr Asp Arg Phe
20 25 30

Arg Gly Pro Asp Ala Leu Cys Arg Ala Val Lys
35 40

<210> SEQ ID NO 251
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCK_A GPCR TM2

<400> SEQUENCE: 251

Thr Asn Ile Phe Leu Leu Ser Leu Ala Val Ser Asp Leu Met Leu Cys
1 5 10 15

Leu Phe Cys Met Pro Phe Asn Leu Ile Pro Asn Leu Leu Lys Asp Phe
20 25 30

Ile Phe Gly Ser Ala Val Cys Lys Thr Thr Thr
35 40

<210> SEQ ID NO 252
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCK_B GPCR TM2

<400> SEQUENCE: 252

Thr Asn Ala Phe Leu Leu Ser Leu Ala Val Ser Asp Leu Leu Leu Ala
1 5 10 15

Val Ala Cys Met Pro Phe Thr Leu Leu Pro Asn Leu Met Gly Thr Phe
20 25 30

Ile Phe Gly Thr Val Ile Cys Lys Ala Val Ser
35 40

<210> SEQ ID NO 253
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: NPY1 GPCR TM2

<400> SEQUENCE: 253

Thr Asn Ile Leu Ile Val Asn Leu Ser Phe Ser Asp Leu Leu Val Ala
1 5 10 15

Ile Met Cys Leu Pro Phe Thr Phe Val Tyr Thr Leu Met Asp His Trp
20 25 30

Val Phe Gly Glu Ala Met Cys Lys Leu Asn Pro
35 40

-continued

<210> SEQ ID NO 254
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: NTR GPCR TM2

<400> SEQUENCE: 254

Val His Tyr His Leu Gly Ser Leu Ala Leu Ser Asp Leu Leu Thr Leu
1 5 10 15

Leu Leu Ala Met Pro Val Glu Leu Tyr Asn Phe Ile Trp Val His His
20 25 30

Pro Trp Ala Phe Gly Asp Ala Gly Cys Arg Gly Tyr Tyr
35 40 45

<210> SEQ ID NO 255
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: NK1 GPCR TM2

<400> SEQUENCE: 255

Thr Asn Tyr Phe Leu Val Asn Leu Ala Phe Ala Glu Ala Ser Met Ala
1 5 10 15

Ala Phe Asn Thr Val Val Asn Phe Thr Tyr Ala Val His Asn Glu Trp
20 25 30

Tyr Tyr Gly Leu Phe Tyr Cys Lys Phe His Asn
35 40

<210> SEQ ID NO 256
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: NK2 GPCR TM2

<400> SEQUENCE: 256

Thr Asn Tyr Phe Ile Val Asn Leu Ala Leu Ala Asp Leu Cys Met Ala
1 5 10 15

Ala Phe Asn Ala Ala Phe Asn Phe Val Tyr Ala Ser His Asn Ile Trp
20 25 30

Tyr Phe Gly Arg Ala Phe Cys Tyr Phe Gln Asn
35 40

<210> SEQ ID NO 257
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: NK3 GPCR TM2

<400> SEQUENCE: 257

Thr Asn Tyr Phe Leu Val Asn Leu Ala Phe Ser Asp Ala Ser Met Ala
1 5 10 15

Ala Phe Asn Thr Leu Val Asn Phe Ile Tyr Ala Leu His Ser Glu Trp
20 25 30

Tyr Phe Gly Ala Asn Tyr Cys Arg Phe Gln Asn
35 40

-continued

<210> SEQ ID NO 258
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: blueops GPCR TM2

<400> SEQUENCE: 258

Leu Asn Tyr Ile Leu Val Asn Val Ser Phe Gly Gly Phe Leu Leu Cys
1 5 10 15

Ile Phe Ser Val Phe Pro Val Phe Val Ala Ser Cys Asn Gly Tyr Phe
20 25 30

Val Phe Gly Arg His Val Cys Ala Leu Glu Gly
35 40

<210> SEQ ID NO 259
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: greenops GPCR TM2

<400> SEQUENCE: 259

Leu Asn Trp Ile Leu Val Asn Leu Ala Val Ala Asp Leu Ala Glu Thr
1 5 10 15

Val Ile Ala Ser Thr Ile Ser Val Val Asn Gln Val Tyr Gly Tyr Phe
20 25 30

Val Leu Gly His Pro Met Cys Val Leu Glu Gly
35 40

<210> SEQ ID NO 260
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: redops GPCR TM2

<400> SEQUENCE: 260

Leu Asn Trp Ile Leu Val Asn Leu Ala Val Ala Asp Leu Ala Glu Thr
1 5 10 15

Val Ile Ala Ser Thr Ile Ser Ile Val Asn Gln Val Ser Gly Tyr Phe
20 25 30

Val Leu Gly His Pro Met Cys Val Leu Glu Gly
35 40

<210> SEQ ID NO 261
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: rhodopsin GPCR TM2

<400> SEQUENCE: 261

Leu Asn Tyr Ile Leu Leu Asn Leu Ala Val Ala Asp Leu Phe Met Val
1 5 10 15

Leu Gly Gly Phe Thr Ser Thr Leu Tyr Thr Ser Leu His Gly Tyr Phe
20 25 30

Val Phe Gly Pro Thr Gly Cys Asn Leu Glu Gly
35 40

-continued

<210> SEQ ID NO 262
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: violetopsGg GPCR TM2

<400> SEQUENCE: 262

Leu Asn Tyr Ile Leu Val Asn Ile Ser Ala Ser Gly Phe Val Ser Cys
1 5 10 15

Val Leu Ser Val Phe Val Val Phe Val Ala Ser Ala Arg Gly Tyr Phe
20 25 30

Val Phe Gly Lys Arg Val Cys Glu Leu Glu Ala
35 40

<210> SEQ ID NO 263
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: opsin_crab GPCR TM2

<400> SEQUENCE: 263

Thr Asn Leu Leu Val Val Asn Leu Ala Phe Ser Asp Phe Cys Met Met
1 5 10 15

Ala Phe Met Met Pro Thr Met Thr Ser Asn Cys Phe Ala Glu Thr Trp
20 25 30

Ile Leu Gly Pro Phe Met Cys Glu Val Tyr Gly
35 40

<210> SEQ ID NO 264
<211> LENGTH: 48
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: ET_Aprec GPCR TM2

<400> SEQUENCE: 264

Pro Asn Ala Leu Ile Ala Ser Leu Ala Leu Gly Asp Leu Ile Tyr Val
1 5 10 15

Val Ile Asp Leu Pro Ile Asn Val Phe Lys Leu Leu Ala Gly Arg Trp
20 25 30

Pro Phe Asp His Asn Asp Phe Gly Val Phe Leu Cys Lys Leu Phe Pro
35 40 45

<210> SEQ ID NO 265
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: ET_Bprec GPCR TM2

<400> SEQUENCE: 265

Pro Asn Ile Leu Ile Ala Ser Leu Ala Leu Gly Asp Leu Leu His Ile
1 5 10 15

Val Ile Asp Ile Pro Ile Asn Val Tyr Lys Leu Leu Ala Glu Asp Trp
20 25 30

Pro Phe Gly Ala Glu Met Cys Lys Leu Val Pro
35 40

-continued

<210> SEQ ID NO 266
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: ET_Cfrog GPCR TM2

<400> SEQUENCE: 266

Pro Asn Val Leu Ile Ala Ser Leu Ala Leu Gly Asp Leu Phe Tyr Ile
1 5 10 15

Leu Ile Ala Ile Pro Ile Ile Ser Ile Ser Phe Trp Leu Ser Thr Gly
20 25 30

His Ser Glu Tyr Ile Tyr Gln
35

<210> SEQ ID NO 267
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: galatin GPCR TM2

<400> SEQUENCE: 267

Thr Asn Leu Phe Ile Leu Asn Leu Ser Ile Ala Asp Leu Ala Tyr Leu
1 5 10 15

Leu Phe Cys Ile Pro Phe Gln Ala Thr Val Tyr Ala Leu Pro Thr Trp
20 25 30

Val Leu Gly Ala Phe Ile Cys Lys Phe Ile His
35 40

<210> SEQ ID NO 268
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: NMBGPCR TM2

<400> SEQUENCE: 268

Pro Asn Ile Phe Ile Ser Asn Leu Ala Ala Gly Asp Leu Leu Leu Leu
1 5 10 15

Leu Thr Cys Val Pro Val Asp Ala Ser Arg Tyr Phe Phe Asp Glu Trp
20 25 30

Met Phe Gly Lys Val Gly Cys Lys Leu Ile Pro
35 40

<210> SEQ ID NO 269
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: GRP GPCR TM2

<400> SEQUENCE: 269

Pro Asn Leu Phe Ile Ser Ser Leu Ala Leu Gly Asp Leu Leu Leu Leu
1 5 10 15

Ile Thr Cys Ala Pro Val Asp Ala Ser Arg Tyr Leu Ala Asp Arg Trp
20 25 30

Leu Phe Gly Arg Ile Gly Cys Lys Leu Ile Pro
35 40

-continued

<210> SEQ ID NO 270
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: BRS3 GPCR TM2

<400> SEQUENCE: 270

Pro Asn Ile Phe Ile Thr Ser Leu Ala Phe Gly Asp Leu Leu Leu Leu
1 5 10 15

Leu Thr Cys Val Pro Val Asp Ala Thr His Tyr Leu Ala Glu Gly Trp
20 25 30

Leu Phe Gly Arg Ile Gly Cys Lys Val Leu Ser
35 40

<210> SEQ ID NO 271
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: deltaOP GPCR TM2

<400> SEQUENCE: 271

Thr Asn Ile Tyr Ile Phe Asn Leu Ala Leu Ala Asp Ala Leu Ala Thr
1 5 10 15

Ser Thr Leu Pro Phe Gln Ser Ala Lys Tyr Leu Met Glu Thr Trp Pro
20 25 30

Phe Gly Glu Leu Leu Cys Lys Ala Val Leu
35 40

<210> SEQ ID NO 272
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: kappaOP GPCR TM2

<400> SEQUENCE: 272

Thr Asn Ile Tyr Ile Phe Asn Leu Ala Leu Ala Asp Ala Leu Val Thr
1 5 10 15

Thr Thr Met Pro Phe Gln Ser Thr Val Tyr Leu Met Asn Ser Trp Pro
20 25 30

Phe Gly Asp Val Leu Cys Lys Ile Val Ile
35 40

<210> SEQ ID NO 273
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: muOP GPCR TM2

<400> SEQUENCE: 273

Thr Asn Ile Tyr Ile Phe Asn Leu Ala Leu Ala Asp Ala Leu Ala Thr
1 5 10 15

Ser Thr Leu Pro Phe Gln Ser Val Asn Tyr Leu Met Gly Thr Trp Pro
20 25 30

Phe Gly Thr Ile Leu Cys Lys Ile Val Ile
35 40

-continued

<210> SEQ ID NO 274
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OPRX GPCR TM2

<400> SEQUENCE: 274

Thr Asn Ile Tyr Ile Phe Asn Leu Ala Leu Ala Asp Thr Leu Val Leu
 1   5   10   15

Leu Thr Leu Pro Phe Gln Gly Thr Asp Ile Leu Leu Gly Phe Trp Pro
            20   25   30

Phe Gly Asn Ala Leu Cys Lys Thr Val Ile
        35   40

<210> SEQ ID NO 275
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CB1 GPCR TM2

<400> SEQUENCE: 275

Ser Tyr His Phe Ile Gly Ser Leu Ala Val Ala Asp Leu Leu Gly Ser
 1   5   10   15

Val Ile Phe Val Tyr Ser Phe Ile Asp Phe His Val Phe His Arg Lys
            20   25   30

Asp Ser Arg Asn Val Phe Leu Phe Lys Leu
        35   40

<210> SEQ ID NO 276
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CB2 GPCR TM2

<400> SEQUENCE: 276

Ser Tyr Leu Phe Ile Gly Ser Leu Ala Gly Ala Asp Phe Leu Ala Ser
 1   5   10   15

Val Val Phe Ala Cys Ser Phe Val Asn Phe His Val Phe His Gly Val
            20   25   30

Asp Ser Lys Ala Val Phe Leu Leu Lys Ile
        35   40

<210> SEQ ID NO 277
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: SSTR1 GPCR TM2

<400> SEQUENCE: 277

Thr Asn Ile Tyr Ile Leu Asn Leu Ala Ile Ala Asp Glu Leu Leu Met
 1   5   10   15

Leu Ser Val Pro Phe Leu Val Thr Ser Thr Leu Leu Arg His Trp Pro
            20   25   30

Phe Gly Ala Leu Leu Cys Arg Leu Val Leu
        35   40

-continued

<210> SEQ ID NO 278
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: SSTR2 GPCR TM2

<400> SEQUENCE: 278

Thr Asn Ile Tyr Ile Leu Asn Leu Ala Ile Ala Asp Glu Leu Phe Met
1 5 10 15

Leu Gly Leu Pro Phe Leu Ala Met Gln Val Ala Leu Val His Trp Pro
20 25 30

Phe Gly Lys Ala Ile Cys Arg Val Val Met
35 40

<210> SEQ ID NO 279
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: SSTR3 GPCR TM2

<400> SEQUENCE: 279

Thr Asn Val Tyr Ile Leu Asn Leu Ala Leu Ala Asp Glu Leu Phe Met
1 5 10 15

Leu Gly Leu Pro Phe Leu Ala Ala Gln Asn Ala Leu Ser Tyr Trp Pro
20 25 30

Phe Gly Ser Leu Met Cys Arg Leu Val Met
35 40

<210> SEQ ID NO 280
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: SSTR4 GPCR TM2

<400> SEQUENCE: 280

Thr Asn Ile Tyr Leu Leu Asn Leu Ala Val Ala Asp Glu Leu Phe Met
1 5 10 15

Leu Ser Val Pro Phe Val Ala Ser Ser Ala Ala Leu Arg His Trp Pro
20 25 30

Phe Gly Ser Val Leu Cys Arg Ala Val Leu
35 40

<210> SEQ ID NO 281
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: SSTR5 GPCR TM2

<400> SEQUENCE: 281

Thr Asn Ile Tyr Ile Leu Asn Leu Ala Val Ala Asp Val Leu Tyr Met
1 5 10 15

Leu Gly Leu Pro Phe Leu Ala Thr Gln Asn Ala Ala Ser Phe Trp Pro
20 25 30

Phe Gly Pro Val Leu Cys Arg Leu Val Met
35 40

-continued

<210> SEQ ID NO 282
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: IL8A and IL8B GPCR TM2

<400> SEQUENCE: 282

Thr Asp Val Tyr Leu Leu Asn Leu Ala Leu Ala Asp Leu Leu Phe Ala
1 5 10 15

Leu Thr Leu Pro Ile Trp Ala Ala Ser Lys Val Asn Gly Trp Ile Phe
20 25 30

Gly Thr Phe Leu Cys Lys Val Val Ser
35 40

<210> SEQ ID NO 283
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AT1a GPCR TM2

<400> SEQUENCE: 283

Ala Ser Val Phe Leu Leu Asn Leu Ala Leu Ala Asp Leu Cys Phe Leu
1 5 10 15

Leu Thr Leu Pro Leu Trp Ala Val Tyr Thr Ala Met Glu Tyr Arg Trp
20 25 30

Pro Phe Gly Asn Tyr Leu Cys Lys Ile Ala Ser
35 40

<210> SEQ ID NO 284
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AT1brat GPCR TM2

<400> SEQUENCE: 284

Ala Ser Val Phe Leu Leu Asn Leu Ala Leu Ala Asp Leu Cys Phe Leu
1 5 10 15

Leu Thr Leu Pro Leu Trp Ala Val Tyr Thr Ala Met Glu Tyr Arg Trp
20 25 30

Pro Phe Gly Asn His Leu Cys Lys Ile Ala Ser
35 40

<210> SEQ ID NO 285
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AT2 GPCR TM2

<400> SEQUENCE: 285

Ser Ser Ile Tyr Ile Phe Asn Leu Ala Val Ala Asp Leu Leu Leu Leu
1 5 10 15

Ala Thr Leu Pro Leu Trp Ala Thr Tyr Tyr Ser Tyr Arg Tyr Asp Trp
20 25 30

Leu Phe Gly Pro Val Met Cys Lys Val Phe Gly
35 40

-continued

<210> SEQ ID NO 286
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: BK1 GPCR TM2

<400> SEQUENCE: 286

Ala Glu Ile Tyr Leu Ala Asn Leu Ala Ala Ser Asp Leu Val Phe Val
1 5 10 15

Leu Gly Leu Pro Phe Trp Ala Glu Asn Ile Trp Asn Gln Phe Asn Trp
20 25 30

Pro Phe Gly Ala Leu Leu Cys Arg Val Ile Asn
35 40

<210> SEQ ID NO 287
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: BK2 GPCR TM2

<400> SEQUENCE: 287

Ala Glu Ile Tyr Leu Gly Asn Leu Ala Ala Ala Asp Leu Ile Leu Ala
1 5 10 15

Cys Gly Leu Pro Phe Trp Ala Ile Thr Ile Ser Asn Asn Phe Asp Trp
20 25 30

Leu Phe Gly Glu Thr Leu Cys Arg Val Val Asn
35 40

<210> SEQ ID NO 288
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P2Y7 GPCR TM2

<400> SEQUENCE: 288

Thr Ala Leu Met Val Leu Asn Leu Ala Leu Ala Asp Leu Ala Val Leu
1 5 10 15

Leu Thr Ala Pro Phe Phe Leu His Phe Leu Ala Gln Gly Thr Trp Ser
20 25 30

Phe Gly Leu Ala Gly Cys Arg Leu Cys His
35 40

<210> SEQ ID NO 289
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P2Y6 GPCR TM2

<400> SEQUENCE: 289

Ser Ala Val Tyr Thr Leu Asn Leu Ala Leu Ala Asp Leu Leu Tyr Ala
1 5 10 15

Cys Ser Leu Pro Leu Leu Ile Tyr Asn Tyr Ala Arg Gly Asp His Trp
20 25 30

Pro Phe Gly Asp Leu Ala Cys Arg Leu Val Arg
35 40

-continued

<210> SEQ ID NO 290
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P2Y5 GPCR TM2

<400> SEQUENCE: 290

Thr Thr Thr Tyr Met Leu Asn Leu Ala Ile Ser Asp Leu Leu Phe Val
1 5 10 15

Phe Thr Leu Pro Phe Arg Ile Tyr Tyr Phe Val Val Arg Asn Trp Pro
20 25 30

Phe Gly Asp Val Leu Cys Lys Ile Ser Val
35 40

<210> SEQ ID NO 291
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P2Y4 GPCR TM2

<400> SEQUENCE: 291

Thr Ala Thr Tyr Met Phe His Leu Ala Leu Ser Asp Thr Leu Tyr Val
1 5 10 15

Val Ser Leu Pro Thr Leu Ile Tyr Tyr Tyr Ala Ala His Asn His Trp
20 25 30

Pro Phe Gly Thr Glu Ile Cys Lys Phe Val Arg
35 40

<210> SEQ ID NO 292
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P2Y3chick GPCR TM2

<400> SEQUENCE: 292

Thr Thr Ile Tyr Met Leu Asn Leu Ala Met Ala Asp Leu Leu Tyr Val
1 5 10 15

Cys Ser Leu Pro Leu Leu Ile Tyr Asn Tyr Thr Gln Lys Asp Tyr Trp
20 25 30

Pro Phe Gly Asp Phe Thr Cys Lys Phe Val Arg
35 40

<210> SEQ ID NO 293
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P2Y2 GPCR TM2

<400> SEQUENCE: 293

Ser Thr Thr Tyr Met Phe His Leu Ala Val Ser Asp Ala Leu Tyr Ala
1 5 10 15

Ala Ser Leu Pro Leu Leu Val Tyr Tyr Tyr Ala Arg Gly Asp His Trp
20 25 30

Pro Phe Ser Thr Val Leu Cys Lys Leu Val Arg
35 40

-continued

<210> SEQ ID NO 294
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P2Y1 GPCR TM2

<400> SEQUENCE: 294

Ile Ser Val Tyr Met Phe Asn Leu Ala Leu Ala Asp Phe Leu Tyr Val
 1   5   10   15

Leu Thr Leu Pro Ala Leu Ile Phe Tyr Tyr Phe Asn Lys Thr Asp Trp
            20   25   30

Ile Phe Gly Asp Ala Met Cys Lys Leu Gln Arg
        35   40

<210> SEQ ID NO 295
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: THRprec GPCR TM2

<400> SEQUENCE: 295

Ala Val Val Tyr Met Leu His Leu Ala Thr Ala Asp Val Leu Phe Val
 1   5   10   15

Ser Val Leu Pro Phe Lys Ile Ser Tyr Tyr Phe Ser Gly Ser Asp Trp
            20   25   30

Gln Phe Gly Ser Glu Leu Cys Arg Phe Val Thr
        35   40

<210> SEQ ID NO 296
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: C5a GPCR TM2

<400> SEQUENCE: 296

Asn Ala Ile Trp Phe Leu Asn Leu Ala Val Ala Asp Phe Leu Ser Cys
 1   5   10   15

Leu Ala Leu Pro Ile Leu Phe Thr Ser Ile Val Gln His His Trp Pro
            20   25   30

Phe Gly Gly Ala Ala Cys Ser Ile Leu Pro
        35   40

<210> SEQ ID NO 297
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: GP01mouse AND R334rat GPCR TM2

<400> SEQUENCE: 297

Met Phe Leu Leu Ile Gly Ser Leu Ala Leu Ala Asp Leu Leu Ala Gly
 1   5   10   15

Leu Gly Leu Ile Ile Asn Phe Val Phe Ala Tyr Leu Leu Gln Ser Glu
            20   25   30

Ala Thr Lys Leu Val Thr Ile
        35

-continued

<210> SEQ ID NO 298
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: GP21mouse GPCR TM2

<400> SEQUENCE: 298

Met Phe Leu Leu Val Gly Ser Leu Ala Val Ala Asp Leu Leu Ala Gly
1 5 10 15

Leu Gly Leu Val Leu His Phe Ala Ala Asp Phe Cys Ile Gly Ser Pro
20 25 30

Glu Met Ser Leu Met Leu Val
35

<210> SEQ ID NO 299
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: GCRCmouse GPCR TM2

<400> SEQUENCE: 299

Thr Ser Leu Phe Ile Val Asn Leu Ala Val Ala Asp Ile Met Ile Thr
1 5 10 15

Leu Leu Asn Thr Pro Phe Thr Leu Val Arg Phe Val Asn Ser Thr Trp
20 25 30

Val Phe Gly Lys Gly Met Cys His Val Ser Arg
35 40

<210> SEQ ID NO 300
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: TXKR GPCR TM2

<400> SEQUENCE: 300

Thr Asn Ser Phe Leu Val Asn Leu Ala Phe Ala Asp Ala Ala Met Ala
1 5 10 15

Ala Leu Asn Ala Leu Val Asn Phe Ile Tyr Ala Leu His Gly Glu Trp
20 25 30

Tyr Phe Gly Ala Asn Tyr Cys Arg Phe Gln Asn
35 40

<210> SEQ ID NO 301
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: G10Drat GPCR TM2

<400> SEQUENCE: 301

Leu Asn Leu Tyr Ile Leu Asn Met Ala Val Ala Asp Leu Gly Ile Ile
1 5 10 15

Leu Ser Leu Pro Val Trp Met Leu Glu Val Met Leu Glu Tyr Thr Trp
20 25 30

Leu Trp Gly Ser Phe Ser Cys Arg Phe Ile His
35 40

-continued

<210> SEQ ID NO 302
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: RDC1 GPCR TM2

<400> SEQUENCE: 302

His Cys Tyr Ile Leu Asn Leu Ala Ile Ala Asp Leu Trp Val Val Leu
 1   5   10   15

Thr Ile Pro Val Trp Val Val Ser Leu Val Gln His Asn Gln Trp Pro
            20   25   30

Met Gly Glu Leu Thr Cys Lys Val Thr His
        35   40

<210> SEQ ID NO 303
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: BLR1 GPCR TM2

<400> SEQUENCE: 303

Thr Phe Leu Phe His Leu Ala Val Ala Asp Leu Leu Leu Val Phe Ile
 1   5   10   15

Leu Pro Phe Ala Val Ala Glu Gly Ser Val Gly Trp Val Leu Gly Thr
            20   25   30

Phe Leu Cys Lys Thr Val Ile
        35

<210> SEQ ID NO 304
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CL5 and LCR1GPCR TM2

<400> SEQUENCE: 304

Leu His Leu Ser Val Ala Asp Leu Leu Phe Val Ile Thr Leu Pro Phe
 1   5   10   15

Trp Ala Val Asp Ala Val Ala Asn Trp Tyr Phe Gly Asn Phe Leu Cys
            20   25   30

Lys Ala Val His
        35

<210> SEQ ID NO 305
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: EBI1 GPCR TM2

<400> SEQUENCE: 305

Leu Leu Asn Leu Ala Val Ala Asp Ile Leu Phe Leu Leu Thr Leu Pro
 1   5   10   15

Phe Trp Ala Tyr Ser Ala Ala Lys Ser Trp Val Phe Gly Val His Phe
            20   25   30

Cys Lys Leu Ile Phe
        35

-continued

<210> SEQ ID NO 306
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: RBS1rat GPCR TM2

<400> SEQUENCE: 306

Leu Leu Asn Leu Ala Leu Ser Asp Leu Leu Phe Val Ala Thr Leu Pro
 1   5   10   15

Phe Trp Thr His Tyr Leu Ile Ser His Glu Gly Leu His Asn Ala Met
            20   25   30

Cys Lys Leu Thr Thr
        35

<210> SEQ ID NO 307
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: EBI2 GPCR TM2

<400> SEQUENCE: 307

Ser Thr Asn Leu Val Ile Ser Asp Ile Leu Phe Thr Thr Ala Leu Pro
 1   5   10   15

Thr Arg Ile Ala Tyr Tyr Ala Met Gly Phe Asp Trp Arg Ile Gly Asp
            20   25   30

Ala Leu Cys Arg Ile Thr Ala
        35

<210> SEQ ID NO 308
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: GCRTchick GPCR TM2

<400> SEQUENCE: 308

Met Leu Asn Leu Ala Ile Ser Asp Leu Leu Phe Val Phe Thr Leu Pro
 1   5   10   15

Phe Arg Ile Tyr Tyr Phe Val Val Arg Asn Trp Pro Phe Gly Asp Val
            20   25   30

Leu Cys Lys Ile Ser Val
        35

<210> SEQ ID NO 309
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: APJ GPCR TM2

<400> SEQUENCE: 309

Ile Phe Ile Ala Ser Leu Ala Val Ala Asp Leu Thr Phe Val Val Thr
 1   5   10   15

Leu Pro Leu Trp Ala Thr Tyr Thr Tyr Arg Asp Tyr Asp Trp Pro Phe
            20   25   30

Gly Thr Phe Phe Cys Lys Leu Ser Ser
        35   40

-continued

<210> SEQ ID NO 310
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: RTArat GPCR TM2

<400> SEQUENCE: 310

Phe Ser Ile Tyr Phe Leu His Leu Ala Ser Ala Asp Gly Ile Tyr Leu
 1   5   10   15

Phe Ser Lys Ala Val Ile Ala Leu Leu Asn Met Gly Thr Phe Leu Gly
            20   25   30

Ser Phe Pro Asp Tyr Val Arg Arg Val Ser Arg
        35   40

<210> SEQ ID NO 311
<211> LENGTH: 44
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: UHRrat GPCR TM2

<400> SEQUENCE: 311

Thr Asn Phe Leu Ile Gly Asn Leu Ala Leu Ser Asp Val Leu Met Cys
 1   5   10   15

Ala Ala Cys Val Pro Leu Thr Leu Ala Tyr Ala Phe Glu Pro Arg Gly
            20   25   30

Trp Val Phe Gly Gly Gly Leu Cys His Leu Val Phe
        35   40

<210> SEQ ID NO 312
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: FMRL1 GPCR TM2

<400> SEQUENCE: 312

Asn Thr Ile Cys Tyr Leu Asn Leu Ala Leu Ala Asp Phe Ser Phe Ser
 1   5   10   15

Ala Ile Leu Pro Phe Arg Met Val Ser Val Ala Met Arg Glu Lys Trp
            20   25   30

Pro Phe Ala Ser Phe Leu Cys Lys Leu Val His
        35   40

<210> SEQ ID NO 313
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: FMRL2 GPCR TM2

<400> SEQUENCE: 313

Thr Thr Ile Cys Tyr Leu Asn Leu Ala Leu Ala Asp Phe Ser Phe Thr
 1   5   10   15

Ala Thr Leu Pro Phe Leu Ile Val Ser Met Ala Met Gly Glu Lys Trp
            20   25   30

Pro Phe Gly Trp Phe Leu Cys Lys Leu Ile His
        35   40

-continued

<210> SEQ ID NO 314
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: fMLP GPCR TM2

<400> SEQUENCE: 314

Thr Thr Ile Ser Tyr Leu Asn Leu Ala Val Ala Asp Phe Cys Phe Thr
1 5 10 15

Ser Thr Leu Pro Phe Phe Met Val Arg Lys Ala Met Gly Gly His Trp
20 25 30

Pro Phe Gly Trp Phe Leu Cys Lys Phe Leu Phe
35 40

<210> SEQ ID NO 315
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLF1catfish GPCR TM2

<400> SEQUENCE: 315

Lys Tyr Ile Thr Val Phe Asn Leu Ala Leu Ser Asp Leu Gly Gly Ser
1 5 10 15

Ser Ala Leu Ile Pro Lys Leu Ile Asp Thr Phe Leu Phe Glu Asn Gln
20 25 30

Val Ile Ser Tyr Glu Ala Cys Leu Ala Asn Met
35 40

<210> SEQ ID NO 316
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLF3catfish GPCR TM2

<400> SEQUENCE: 316

Lys Tyr Ile Ala Val Phe Asn Leu Ala Leu Ser Asp Leu Cys Gly Ser
1 5 10 15

Ser Ala Leu Ile Pro Lys Leu Leu Asp Met Leu Leu Phe Glu Asn Gln
20 25 30

Ser Ile Ser Tyr Glu Ala Cys Leu Ser Asn Met
35 40

<210> SEQ ID NO 317
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLF8catfish GPCR TM2

<400> SEQUENCE: 317

Met Cys Ile Leu Ile Gly Leu Met Ala Val Val Asp Leu Ser Met Pro
1 5 10 15

Ile Phe Cys Val Pro Asn Met Leu Leu Ser Phe Leu Phe Asn Trp Lys
20 25 30

Gly Ile Ser Leu Val Gly Cys Leu Val Gln Met
35 40

-continued

<210> SEQ ID NO 318
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLF32Acatfish GPCR TM2

<400> SEQUENCE: 318

Lys Tyr Met Gly Ile Phe Asn Leu Ala Leu Ser Asp Phe Gly Glu Thr
 1   5   10   15

Asn Val Leu Ile Pro Ser Leu Val Lys Thr Leu Phe Phe Asp Ser Gln
            20   25   30

Tyr Ile Ser Tyr Asp Ala Cys Leu Ala Asn Met
        35   40

<210> SEQ ID NO 319
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLF32Bcatfish and OLF32Dcatfish GPCR TM2

<400> SEQUENCE: 319

Lys Tyr Met Gly Ile Phe Asn Leu Ala Leu Ser Asp Phe Gly Glu Thr
 1   5   10   15

Asn Ala Leu Ile Pro Ser Leu Val Lys Thr Leu Phe Phe Asp Ser Gln
            20   25   30

Tyr Ile Ser Tyr Asp Ala Cys Leu Ala Asn Met
        35   40

<210> SEQ ID NO 320
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLF32Ccatfish GPCR TM2

<400> SEQUENCE: 320

Lys Tyr Met Gly Ile Phe Asn Leu Ala Leu Ser Asp Ile Gly Glu Thr
 1   5   10   15

Asn Ala Leu Ile Pro Ser Leu Val Lys Thr Leu Phe Phe Asp Ser Gln
            20   25   30

Tyr Ile Ser Tyr Asp Ala Cys Leu Thr Asn Met
        35   40

<210> SEQ ID NO 321
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLF47catfish GPCR TM2

<400> SEQUENCE: 321

Lys Phe Leu Ala Val Phe Asn Leu Ala Val Val Asp Ile Ser Ile Asn
 1   5   10   15

Ser Val Ile Ile Pro Gln Met Val Pro Val Phe Val Phe Asn Leu Asn
            20   25   30

His Ile Ser Phe Glu Ser Cys Phe Ser Gln Met
        35   40

-continued

<210> SEQ ID NO 322
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLF202catfish GPCR TM2

<400> SEQUENCE: 322

Met Tyr Tyr Ile Met Leu Asn Leu Ala Ala Ser Asp Val Leu Phe Ser
 1   5   10   15

Thr Thr Thr Leu Pro Lys Ile Ile Ala Arg Tyr Trp Phe Gly Asp Gly
            20   25   30

Ser Ile Ser Phe Val Gly Cys Phe Ile Gln Met
        35   40

<210> SEQ ID NO 323
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFCOR1chicken, OLFCOR3chicken AND
      OLFCOR4chicken GPCR TM2

<400> SEQUENCE: 323

Met Tyr Ile Phe Leu Gln Asn Leu Ser Phe Thr Asp Ala Ala Tyr Ser
 1   5   10   15

Thr Val Ile Thr Pro Lys Met Leu Ala Thr Phe Leu Glu Glu Arg Lys
            20   25   30

Thr Ile Ser Tyr Val Gly Cys Ile Leu Gln Tyr
        35   40

<210> SEQ ID NO 324
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFCOR2chicken and OLFCOR5chicken GPCR TM2

<400> SEQUENCE: 324

Met Tyr Ile Phe Leu Gln Asn Leu Ser Phe Thr Asp Ala Ala Tyr Ser
 1   5   10   15

Thr Val Ile Thr Pro Lys Met Leu Ala Thr Phe Leu Glu Glu Arg Arg
            20   25   30

Thr Ile Ser Tyr Val Gly Cys Ile Leu Gln Tyr
        35   40

<210> SEQ ID NO 325
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFCOR6chicken GPCR TM2

<400> SEQUENCE: 325

Met Tyr Ile Phe Leu Gln Asn Leu Ser Phe Thr Asp Ala Val Tyr Ser
 1   5   10   15

Thr Val Ile Thr Pro Lys Met Leu Ala Thr Phe Leu Glu Glu Thr Lys
            20   25   30

Thr Ile Ser Tyr Val Gly Cys Ile Leu Gln Tyr
        35   40

-continued

<210> SEQ ID NO 326
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFdog GPCR TM2

<400> SEQUENCE: 326

Met Tyr Leu Phe Leu Ser Asn Leu Ser Phe Ser Asp Leu Cys Phe Ser
 1   5   10   15

Ser Val Thr Met Pro Lys Leu Leu Gln Asn Met Gln Ser Gln Val Pro
            20   25   30

Ser Ile Pro Tyr Ala Gly Cys Leu Thr Gln Met
        35   40

<210> SEQ ID NO 327
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLF07E GPCR TM2

<400> SEQUENCE: 327

Val Tyr Phe Phe Leu Ala Asn Leu Ser Phe Thr Asp Leu Phe Phe Val
 1   5   10   15

Thr Asn Thr Ile Pro Lys Met Leu Val Asn Leu Gln Ser His Asn Lys
            20   25   30

Ala Ile Ser Tyr Ala Gly Cys Leu Thr Gln Leu
        35   40

<210> SEQ ID NO 328
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLF07I GPCR TM2

<400> SEQUENCE: 328

Met Tyr Leu Phe Leu Ser Asn Leu Ser Phe Ser Asp Leu Cys Phe Ser
 1   5   10   15

Ser Val Thr Ile Pro Lys Leu Leu Gln Asn Met Gln Asn Gln Asp Pro
            20   25   30

Ser Ile Pro Tyr Ala Asp Cys Leu Thr Gln Met
        35   40

<210> SEQ ID NO 329
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLF07J GPCR TM2

<400> SEQUENCE: 329

Met Tyr Phe Phe Leu Ser Met Leu Ser Thr Ser Glu Thr Val Tyr Thr
 1   5   10   15

Leu Val Ile Leu Pro Arg Met Leu Ser Ser Leu Val Gly Met Ser Gln
            20   25   30

Pro Met Ser Leu Ala Gly Cys Ala Thr Gln Met
        35   40

-continued

<210> SEQ ID NO 330
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFOR3mouse GPCR TM2

<400> SEQUENCE: 330

Met Tyr Phe Phe Leu Ser Asn Leu Ser Ser Leu Asp Leu Ala Phe Thr
1 5 10 15

Thr Ser Ser Val Pro Gln Met Leu Lys Asn Leu Trp Gly Pro Asp Lys
20 25 30

Thr Ile Ser Tyr Gly Gly Cys Val Thr Gln Leu
35 40

<210> SEQ ID NO 331
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFrat GPCR TM2

<400> SEQUENCE: 331

Met Tyr Tyr Phe Leu Ser Ser Leu Ser Phe Val Asp Leu Cys Tyr Ser
1 5 10 15

Thr Val Ile Thr Pro Lys Met Leu Val Asn Phe Leu Gly Lys Lys Asn
20 25 30

Phe Ile Thr Tyr Ser Glu Cys Met Ala Gln Phe
35 40

<210> SEQ ID NO 332
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFF3rat GPCR TM2

<400> SEQUENCE: 332

Met Tyr Phe Phe Leu Ser Asn Leu Ser Phe Val Asp Ile Cys Phe Ile
1 5 10 15

Ser Thr Thr Val Pro Lys Met Leu Val Asn Ile Gln Thr Gln Asn Asn
20 25 30

Val Ile Thr Tyr Ala Gly Cys Ile Thr Gln Ile
35 40

<210> SEQ ID NO 333
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFF5rat GPCR TM2

<400> SEQUENCE: 333

Met Tyr Phe Phe Leu Ser Asn Leu Ser Phe Val Asp Val Cys Phe Ser
1 5 10 15

Ser Thr Thr Val Pro Lys Val Leu Ala Asn His Ile Leu Gly Ser Gln
20 25 30

Ala Ile Ser Phe Ser Gly Cys Leu Thr Gln Leu
35 40

-continued

<210> SEQ ID NO 334
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFF6rat GPCR TM2

<400> SEQUENCE: 334

Met Tyr Phe Phe Leu Cys Asn Leu Ser Phe Leu Glu Ile Trp Phe Thr
 1   5   10   15

Thr Ala Cys Val Pro Lys Thr Leu Ala Thr Phe Ala Pro Arg Gly Gly
            20   25   30

Val Ile Ser Leu Ala Gly Cys Ala Thr Gln Met
        35   40

<210> SEQ ID NO 335
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFF12rat GPCR TM2

<400> SEQUENCE: 335

Met Tyr Phe Phe Leu Ala Asn Leu Ser Phe Val Asp Ile Cys Phe Thr
 1   5   10   15

Ser Thr Thr Ile Pro Lys Met Leu Val Asn Ile Tyr Thr Gln Ser Lys
            20   25   30

Ser Ile Thr Tyr Glu Asp Cys Ile Ser Gln Met
        35   40

<210> SEQ ID NO 336
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFI3rat GPCR TM2

<400> SEQUENCE: 336

Met Tyr Leu Phe Leu Ser Asn Leu Ser Phe Ser Asp Leu Cys Phe Ser
 1   5   10   15

Ser Val Thr Met Pro Lys Leu Leu Gln Asn Met Arg Ser Gln Asp Thr
            20   25   30

Ser Ile Pro Tyr Gly Gly Cys Leu Ala Gln Thr
        35   40

<210> SEQ ID NO 337
<211> LENGTH: 47
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFI7rat GPCR TM2

<400> SEQUENCE: 337

Met Tyr Phe Phe Leu Ala Asn Met Ser Phe Leu Glu Ile Trp Tyr Val
 1   5   10   15

Thr Val Thr Ile Pro Lys Met Leu Ala Gly Phe Ile Gly Ser Lys Glu
            20   25   30

Asn His Gly Gln Leu Ile Ser Phe Glu Ala Cys Met Thr Gln Leu
        35   40   45

-continued

<210> SEQ ID NO 338
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFI8rat GPCR TM2

<400> SEQUENCE: 338

Met Tyr Leu Phe Leu Ser Asn Leu Ser Phe Ser Asp Leu Cys Phe Ser
1 5 10 15

Ser Val Thr Met Leu Lys Leu Leu Gln Asn Ile Gln Ser Gln Val Pro
20 25 30

Ser Ile Ser Tyr Ala Gly Cys Leu Thr Gln Ile
35 40

<210> SEQ ID NO 339
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFI9rat GPCR TM2

<400> SEQUENCE: 339

Met Tyr Leu Phe Leu Ser Asn Leu Ser Phe Ala Asp Leu Cys Phe Ser
1 5 10 15

Ser Val Thr Met Pro Lys Leu Leu Gln Asn Met Gln Ser Gln Val Pro
20 25 30

Ser Ile Pro Tyr Ala Gly Cys Leu Ala Gln Ile
35 40

<210> SEQ ID NO 340
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFI14rat GPCR TM2

<400> SEQUENCE: 340

Met Tyr Leu Phe Leu Ser Asn Leu Ser Phe Ser Asp Leu Cys Phe Ser
1 5 10 15

Ser Val Thr Met Pro Lys Leu Leu Gln Asn Met Gln Ser Gln Val Pro
20 25 30

Ser Ile Ser Tyr Thr Gly Cys Leu Thr Gln Leu
35 40

<210> SEQ ID NO 341
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFI15rat GPCR TM2

<400> SEQUENCE: 341

Met Tyr Leu Phe Leu Ser Asn Leu Ser Phe Ser Asp Leu Cys Phe Ser
1 5 10 15

Ser Val Thr Met Pro Lys Leu Leu Gln Asn Met Gln Ser Gln Val Pro
20 25 30

Ser Ile Pro Phe Ala Gly Cys Leu Thr Gln Leu
35 40

-continued

<210> SEQ ID NO 342
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OLFOR17_40 GPCR TM2

<400> SEQUENCE: 342

Met Tyr Phe Phe Leu Gly Asn Leu Ser Val Leu Asp Val Gly Cys Ile
1 5 10 15

Ser Val Thr Val Pro Ser Met Leu Ser Arg Leu Leu Ser Arg Lys Arg
20 25 30

Ala Val Pro Cys Gly Ala Cys Leu Thr Gln Leu
35 40

<210> SEQ ID NO 343
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: GUST27rat GPCR TM2

<400> SEQUENCE: 343

Met Tyr Phe Phe Leu Ser Asn Leu Ser Phe Val Asp Ile Cys Phe Ile
1 5 10 15

Ser Thr Thr Ile Pro Lys Met Leu Val Asn Ile His Ser Gln Thr Lys
20 25 30

Asp Ile Ser Tyr Ile Glu Cys Leu Ser Gln Val
35 40

<210> SEQ ID NO 344
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: RPE GPCR TM2

<400> SEQUENCE: 344

Cys His Leu Leu Val Leu Ser Leu Ala Leu Ala Asp Ser Gly Ile Ser
1 5 10 15

Leu Asn Ala Leu Val Ala Ala Thr Ser Ser Leu Leu Arg Arg Trp Pro
20 25 30

Tyr Gly Ser Asp Gly Cys Gln Ala His Gly
35 40

<210> SEQ ID NO 345
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: HHRF1 GPCR TM2

<400> SEQUENCE: 345

Gly Asp Val Tyr Phe Ile Asn Leu Ala Ala Ala Asp Leu Leu Phe Val
1 5 10 15

Cys Thr Leu Pro Leu Trp Met Gln Tyr Leu Leu Asp His Asn Ser Leu
20 25 30

Ala Ser Val Pro Cys Thr Leu Leu Thr
35 40

-continued

<210> SEQ ID NO 346
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: HHRF2 GPCR TM2

<400> SEQUENCE: 346

Ser Asp Thr Tyr Ile Cys Asn Leu Ala Val Ala Asp Leu Leu Ile Val
 1   5   10   15

Val Gly Leu Pro Phe Phe Leu Glu Tyr Ala Lys His His Pro Lys Leu
            20   25   30

Ser Arg Glu Val Val Cys Ser Gly Leu Asn
        35   40

<210> SEQ ID NO 347
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: HHRF3 GPCR TM2

<400> SEQUENCE: 347

Pro Thr Ile Tyr Met Thr Asn Leu Tyr Ser Thr Asn Phe Leu Thr Leu
 1   5   10   15

Thr Val Leu Pro Phe Ile Val Leu Ser Asn Gln Trp Leu Leu Pro Ala
            20   25   30

Gly Val Ala Ser Cys Lys Phe Leu Ser
        35   40

<210> SEQ ID NO 348
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: MCP-1A and MCP-1B GPCR TM2

<400> SEQUENCE: 348

Thr Asp Ile Tyr Leu Leu Asn Leu Ala Ile Ser Asp Leu Leu Phe Leu
 1   5   10   15

Ile Thr Leu Pro Leu Trp Ala His Ser Ala Ala Asn Glu Trp Val Phe
            20   25   30

Gly Asn Ala Met Cys Lys Leu Phe Thr
        35   40

<210> SEQ ID NO 349
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PPR1bovine GPCR TM2

<400> SEQUENCE: 349

Thr Asp Val Tyr Ile Leu Asn Leu Ala Val Ala Asp Leu Phe Leu Leu
 1   5   10   15

Phe Thr Leu Pro Phe Trp Ala Val Asn Ala Val His Gly Trp Val Leu
            20   25   30

Gly Lys Ile Met Cys Lys Val Thr Ser
        35   40

-continued

<210> SEQ ID NO 350
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: F-1-5 GPCR CXCR4
<221> NAME/KEY: MOD_RES
<222> LOCATION: (18)...(18)
<223> OTHER INFORMATION: Xaa = valinamide

<400> SEQUENCE: 350

Asp Asp Ile Phe Leu Pro Thr Ile Tyr Ser Ile Ile Phe Leu Thr Gly
1 5 10 15

Ile Xaa

<210> SEQ ID NO 351
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: F-2-1 GPCR CXCR4

<400> SEQUENCE: 351

Leu Leu Phe Val Ile Thr Leu Pro Phe Trp Ala Val Asp Ala Val Ala
1 5 10 15

Asn Trp Tyr Phe Gly Asn
20

<210> SEQ ID NO 352
<211> LENGTH: 23
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: F-3-1 GPCR CXCR4
<221> NAME/KEY: MOD_RES
<222> LOCATION: (23)...(23)
<223> OTHER INFORMATION: Xaa = leucinamide

<400> SEQUENCE: 352

Lys Ala Val His Val Ile Tyr Thr Val Asn Leu Tyr Ser Ser Val Leu
1 5 10 15

Ile Leu Ala Phe Ile Ser Xaa
20

<210> SEQ ID NO 353
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: F-4-1 GPCR CXCR4

<400> SEQUENCE: 353

Lys Val Tyr Val Gly Val Trp Ile Pro Ala Leu Leu Leu Thr Ile Pro
1 5 10 15

Asp Phe Ile Phe
20

<210> SEQ ID NO 354
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: F-5-1 GPCR CXCR4
<221> NAME/KEY: MOD_RES
<222> LOCATION: (21)...(21)
<223> OTHER INFORMATION: Xaa = isoleucinamide

-continued

<400> SEQUENCE: 354

His Ile Met Val Gly Leu Ile Leu Pro Gly Ile Val Ile Leu Ser Cys
 1   5   10   15

Tyr Cys Ile Ile Xaa
            20

<210> SEQ ID NO 355
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: F-7-1 GPCR CXCR4
<221> NAME/KEY: MOD_RES
<222> LOCATION: (20)...(20)
<223> OTHER INFORMATION: Xaa = lysinamide

<400> SEQUENCE: 355

Ala Leu Ala Phe Phe His Cys Cys Leu Asn Pro Ile Leu Tyr Ala Phe
 1   5   10   15

Leu Gly Ala Xaa
            20

<210> SEQ ID NO 356
<211> LENGTH: 32
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: second transmembrane domain of CXCR4

<400> SEQUENCE: 356

His Leu Ser Val Ala Asp Leu Leu Phe Val Ile Thr Leu Pro Phe Trp
 1   5   10   15

Ala Val Asp Ala Val Ala Asn Trp Tyr Phe Gly Asn Phe Leu Cys Lys
            20   25   30

<210> SEQ ID NO 357
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: F-2-8 GPCR CXCR4

<400> SEQUENCE: 357

Leu Leu Phe Val Ile Thr Leu Pro Phe Trp Ala Val Asp Ala Val Ala
 1   5   10   15

Asn Trp Tyr Phe Gly Asn Lys Lys
            20

<210> SEQ ID NO 358
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: F-2-4 GPCR CXCR4

<400> SEQUENCE: 358

Val Ile Thr Leu Pro Phe Trp Ala Val Asp Ala Val Ala Asn Trp Tyr
 1   5   10   15

Phe Gly Asn Lys Lys
            20

-continued

<210> SEQ ID NO 359
<211> LENGTH: 19
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AcF-2-5 GPCR CXCR4
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)...(1)
<223> OTHER INFORMATION: Xaa = acetylated Leu

<400> SEQUENCE: 359

Xaa Leu Phe Val Ile Thr Leu Pro Phe Trp Ala Val Asp Ala Val Ala
1 5 10 15

Asn Asp Asp

<210> SEQ ID NO 360
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: F-2-6 GPCR CXCR4

<400> SEQUENCE: 360

Leu Ser Val Ala Asp Leu Leu Phe Val Ile Thr Leu Pro Phe Trp Ala
1 5 10 15

Val Asp Ala Val Ala Asn Asp Asp
20

<210> SEQ ID NO 361
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Rhod-AcF-2-2 GPCR CXCR4
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)...(1)
<223> OTHER INFORMATION: Xaa = acetylated Leu
<221> NAME/KEY: MOD_RES
<222> LOCATION: (26)...(26)
<223> OTHER INFORMATION: Xaa = rhodamine linked to Lys

<400> SEQUENCE: 361

Xaa Leu Leu Phe Val Ile Thr Leu Pro Phe Trp Ala Val Asp Ala Val
1 5 10 15

Ala Asn Trp Tyr Phe Gly Asn Asp Asp Xaa Asp
20 25

<210> SEQ ID NO 362
<211> LENGTH: 28
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCKAR-TM-4-2 (#71) GPCR CCKAR

<400> SEQUENCE: 362

Val Ile Ala Ala Thr Trp Cys Leu Ser Phe Thr Ile Met Thr Pro Tyr
1 5 10 15

Pro Ile Tyr Ser Asn Leu Val Pro Phe Thr Asp Asp
20 25

<210> SEQ ID NO 363
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CCKAR-TM-5-3 (#45) GPCR CCKAR

-continued

<400> SEQUENCE: 363

Asp Asp Gln Thr Phe Leu Leu Leu Ile Leu Phe Leu Leu Pro Gly Ile
1 5 10 15

Val Met Val Val Ala Tyr Gly Leu
20

What is claimed is:

1. A method of inhibiting the biological activity of a target G protein-coupled receptor (GPCR) by contacting a cell that expresses said GPCR with an isolated GPCR-modulating molecule comprising a peptide that has at least five amino acids, an N-terminus and a C-terminus, said at least five amino acids being identical to the amino acid sequence of a portion of a transmembrane domain of said GPCR, wherein

(a) said molecule has an extracellular end group that is negatively charged under physiological conditions, an intracellular end group with a neutral charge sufficient to allow insertion into a membrane under physiological conditions, and said molecule comprises the following modifications: when said intracellular end group is said C-terminus, said neutral charge includes a terminal carboxylamide group; or, when said intracellular end group is said N-terminus, said peptide or peptidomimetic includes at least four of the first five N-terminal amino acids having neutrally-charged amino acid side chains under physiologic conditions; and at least one of the end groups is a group that does not naturally occur at that position in the transmembrane sequence;

(b) said molecule spontaneously inserts into a membrane in the same orientation as the transmembrane domain from which it is derived, wherein said molecule without the modifications in (a) does not spontaneously insert into the membrane in said orientation; and

(c) said molecule inhibits a biological activity of said GPCR by disrupting the structure or assembly of said GPCR,

wherein the biological activity is selected from the group consisting of signal transduction, binding of a virus and subsequent infection, tumor growth, chemotaxis, mitogenic response, cell growth activation, secretion, muscle contraction, vasopressing and vasodepressing activity, synaptic transmission, and release of intracellular calcium.

2. A method of claim **1**, wherein the concentration of the molecule is about 0.01 to about 100 micromolar.

3. A method of claim **1**, wherein the molecule inhibits a signal transduction of the GPCR.

4. A method of claim **1**, wherein the molecule comprises a peptide selected from the group consisting of:

From the GPCR CXCR4
F-2-2: LLFVITLPFWAVDAVANWYFGNDD (SEQ ID NO:1)

F-2-5: LLFVITLPFWAVDAVANDD-OH (SEQ ID NO:2)

F-4-2: VYVGVWIPALLLTIPDFIFANDD-OH (SEQ ID NO:3)

F-6-1: VILILAFFACWLPYYIGISID-OH (SEQ ID NO:4)

F-7-3: DDEALAFFHCCLNPILYAFL-NH_2 (SEQ ID NO:5)

F-7-4: DDSITEALAFFHCCLNPILYAFL-NH_2 (SEQ ID NO:6)

From the GPCR CCR5
CCR5-TM-2-2: LFFL LTVPFWAHYAAAQWDFGDD (SEQ ID NO:7)

CCR5-TM-4-1: FGVVTSVITWVVAVFASLPGIIFTSSDD (SEQ ID NO:8)

CCR5-TM-6-1: LIFTIMIVYFLFWAPYNIVLLLNTFQED (SEQ ID NO:9)

From the GPCR CCR2
CCR2-TM-2-1: IYLLNLAISDLLFLITLPLWADD-OH (SEQ ID NO:11)

CCR2-TM-2-2: LLFLITLPLWAH SAANEWVFGNDD-OH (SEQ ID NO:12)

CCR2-TM-4-1: FGVVTSVITWLVAVF ASVPGIIFTDD (SEQ ID NO:13)

CCR2-TM-6-1: VIFTIMIVYFLFWTPYN IVILLNTFQED (SEQ ID NO:14)

From the GPCR CCR3
CCR3-TM-2-1: LLFLVTLPFW IHYVRGHNWVFGDDD (SEQ ID NO:16)

CCR3-TM-4-1: FGVITSIVTWGLAVLAALPEFI FYETED (SEQ ID NO:17)

CCR3-TM-6-1: IFVIMAVFFI FWTPYNVAILLSSYQSDD (SEQ ID NO:18)

-continued

From the GPCR CCKAR
CCKAR-TM-2-2: FLLSLAVSDLMLCLFCM PFNLIDD (SEQ ID NO:22)

CCKAR-TM-6-4: IVVLFFLCWMPIFSANAWRAYDTVDD (SEQ ID NO:23).

5. A method of claim **1**, wherein the target GPCR is CCKAR and the inhibited biological activity is inhibition of CCKAR-mediated intracellular Ca^{2+} release.

6. A method of inhibiting the biological activity of a target G protein-coupled receptor (GPCR) by contacting a cell that expresses said GPCR with an isolated GPCR-modulating molecule comprising a peptide that has at least five amino acids, an N-terminus and a C-terminus, said at least five amino acids being identical to the amino acid sequence of a portion of a transmembrane domain of said GPCR, wherein

(a) said molecule has an extracellular end group that is negatively charged under physiological conditions, an intracellular end group with a neutral charge sufficient to allow insertion into a membrane under physiological conditions, and said molecule comprises the following modifications: said intracellular end group is said C-terminus and said neutral charge includes a terminal carboxylamide group; and at least one of the end groups is a group that does not naturally occur at that position in the transmembrane sequence;

(b) said molecule spontaneously inserts into a membrane in the same orientation as the transmembrane domain from which it is derived, wherein said molecule without the modifications in (a) does not spontaneously insert into the membrane in said orientation; and

(c) said molecule inhibits a biological activity of said GPCR by disrupting the structure or assembly of said GPCR,

wherein the biological activity is selected from the group consisting of signal transduction, binding of a virus and subsequent infection, tumor growth, chemotaxis, mitogenic response, cell growth activation, secretion, muscle contraction, vasopressing and vasodepressing activity, synaptic transmission, and release of intracellular calcium.

7. The method of claim **6**, wherein the molecule comprises a peptide selected from the group consisting of:

From the GPCR CXCR4
F-7-3: DDEALAFFHCCLNPILYAFL-NH_2 (SEQ ID NO:5)

F-7-4: DDSITEALAFFHCCLNPILYAFL-NH_2 (SEQ ID NO:6).

* * * * *